Replacement of domain b of human protein disulfide isomerase-related protein with domain b′ of human protein disulfide isomerase dramatically increases its chaperone activity

Tomohisa Horibe, Daisuke Iguchi, Toshio Masuoka, Mitsuhiro Gomi, Taiji Kimura, Masakazu Kikuchi*

Department of Bioscience and Technology, Faculty of Science and Engineering, Ritsumeikan University, 1-1-1 Noji-higashi, Kusatsu, Shiga, 525-8577, Japan

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Abstract We have reported that human protein disulfide isomerase-related protein (hPDIR) has isomerase and chaperone activities that are lower than those of the human protein disulfide isomerase (hPDI), and that the b domain of hPDIR is critical for its chaperone activity [J. Biol. Chem. 279 (2004) 4604]. To investigate the basis of the differences between hPDI and hPDIR, and to determine the functions of each hPDIR domain in detail, we constructed several hPDIR domain mutants. Interestingly, when the b domain of hPDIR was replaced with the b′ domain of hPDI, a dramatic increase in chaperone activity that was close to that of hPDI itself was observed. However, this mutant showed decreased oxidative refolding of α1-antitrypsin. The replacement of the b domain of hPDIR with the c domain of hPDI also increased its chaperone activity. These observations suggest that putative peptide-binding sites of hPDI determine both its chaperone activity and its substrate specificity.

Keywords: Protein disulfide isomerase; Protein disulfide isomerase-related protein; Chaperone activity; Domain mutation; CXXC motif

1. Introduction

Protein disulfide isomerase (PDI) catalyzes the oxidation, reduction, and isomerization of protein disulfide bonds [1]. It is believed to accelerate the folding of disulfide-bonded proteins by catalyzing the disulfide interchange reaction, which is the rate-limiting step of protein folding that occurs within the luminal space of the endoplasmic reticulum (ER) [2–4]. PDI has two distinct regions that contain the Cys–Gly–His–Cys (CGHC) sequence. This sequence is similar to a motif found in thioredoxin, namely, Cys–Gly–Pro–Cys (CGPC), which is also called the TX motif or CXXC motif. The CGHC sequences serve as the active sites in PDI for thiol-disulfide bond exchange reactions [5]. In addition to catalyzing protein folding, PDI functions as a β-subunit of prolyl 4-hydroxylase [6] and as a component of triglyceride transfer protein [7]. It is also essential for yeast viability [8–10]. Moreover, it has recently been reported that PDI binds several drugs such as aminoglycoside antibiotics [11,12] and copper [13], and that PDI is a major calcium-binding protein in the ER [14,15]. However, the detailed physiological roles that are played by these multiple cellular functions of PDI still remain to be elucidated.

We recently reported that human protein disulfide isomerase-related protein (hPDIR), which has three CXXC motifs, namely, Cys–Ser–Met–Cys (CSMC), CGHC, and Cys–Pro–His–Cys (CPHC), also has isomerase and chaperone activities, although both activities were about 2% and 50% of those of human protein disulfide isomerase (hPDI) [16]. We also showed that hPDIR bears an oxidative refolding activity that is specific for α1-antitrypsin [16].

hPDI and hPDIR have been proposed to bear the a–b–b′–a′–c and b–a^0–a–a′ domain structures, respectively [17]. The a^0, a, and a′ regions are redox-active thioredoxin domains, while b and b′ are redox-inactive thioredoxin domains, and c is a highly acidic region. Two of the CGHC sequences of PDI are located in the a and a′ domains, while the CXXC motifs of hPDIR are in the a^0, a, and a′ domains [17]. A candidate peptide-binding site is located in the b′ and c domains of PDI [17], and little overall amino acid homology between hPDI and hPDIR is observed. We found previously that the b domain of hPDIR is crucial for its chaperone activity but not for its isomerase activity, and that the chaperone activity of wild-type hPDIR is approximately 50% that of the wild-type hPDI [16].

Here, we show that the replacement of the b domain of hPDIR with the b′ domain of hPDI dramatically increases its chaperone activity. The functions of each domain of hPDIR and hPDI are also discussed.

2. Materials and methods

2.1. Materials

Rhodanese, human α1-antitrypsin and *N*-succinyl–Ala–Ala–Pro–Phe-*p*-nitroanilide were purchased from Sigma Chemical Co. (St.

* Corresponding author. Present address: Kobayashi Pharmaceutical Co., Ltd., R&D Company, 30-3, 1-Chome, Toyokawa, Ibaraki-Shi, Osaka 567-0057, Japan. Fax: +81-77-561-2659.
E-mail address: kikuchi@se.ritsumei.ac.jp (M. Kikuchi).

Abbreviations: hPDI, human protein disulfide isomerase; hPDIR, human protein disulfide isomerase-related protein; ER, endoplasmic reticulum; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electorophoresis; GSH, glutathione (reduced form); GSSG, glutathione (oxidized form)

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Louis, USA). A Hitrap SP FPLC column was purchased from Amersham Biosciences (Uppsala, Sweden). α-Chymotrypsin and other reagents were mostly obtained from Wako Pure Chemical Industries (Osaka, Japan). All reagents were of research grade quality.

2.2. Strains and plasmids

Escherichia coli AD494 (DE3) {Δ*ara*, *leu* 7697, Δ*lac*X74, Δ*pho*A, *Pvu*II, *Pho*R, Δ*mal*F3, F′ [*lac*$^-$, (*lacI*^{q}), *pro*], *trx*B::*kan* (DE3)} and pET-15b (Novagen, Inc., Madison, USA) were used to express hPDIR, hPDI, and the hPDIR domain mutants.

2.3. Expression and purification of hPDIR, hPDI, and domain mutants of hPDIR

Expression plasmids encoding hPDIR, hPDI, and hPDIR domain mutants were constructed using pET-15b according to the methods described previously [16,18]. The expression and the purification of these proteins using a Ni^{2+}-chelating resin column were also performed as described previously [16,18]. The proteins were purified further with a Hitrap SP FPLC column after dialysis against 20 mM sodium phosphate buffer (pH 7.12), and the proteins were eluted with a linear gradient of 0–1 M NaCl. The purity of the proteins was determined by sodium dodecyl sulfate–polyacrylamide gel electorophoresis (SDS–PAGE), which was performed according to the method of Laemmli [19]. The protein concentration was quantified using UV absorption method [20].

2.4. Assays of the activities of hPDIR, hPDI, and hPDIR domain mutants

Isomerase activity was determined according to the method of Lambert and Freedman [21]. This method is based on the GSH-catalyzed reduction of the disulfide bonds in insulin that is associated with the reduction of GSSG to GSH, which is mediated by NADPH and glutathione reductase. To discriminate between isomerase and chaperone activities, rhodanese, which does not contain disulfide bonds, was used as a substrate for chaperone activity. Chaperone activity was evidenced by the prevention of denatured rhodanese aggregation and was assayed according to the methods of Martin et al. [22]. The oxidative refolding of α1-antitrypsin was determined as described previously [16]. In all experiments, purified wild type hPDIR and hPDI were used as controls.

2.5. Measuring the intrinsic fluorescence emission spectra of hPDIR, hPDI, and hPDIR domain mutants

Fluorescence spectra were collected on a Hitachi Spectrofluorometer F-4010 using a 3 ml cuvette. All scans were collected at 25 °C as an average of three scans with excitation of 280 nm, emission of 300–400 nm, slit widths of 5 nm, and scan speed of 50 nm/min. All spectra were corrected for the blank spectra with no protein added.

2.6. Secondary structure prediction

The amino acid sequences of the hPDIR b domain (2–115), the hPDI b domain (118–217), and the hPDI b′ domain (218–351) were analyzed by several different algorithms on a protein secondary prediction server. One algorithm, SSTHREAD (http://www.ddbj.nig.ac.jp/E-mail/ssthread/welcome.html), yielded a secondary structure of the hPDI b domain that fitted particularly well to the secondary structure that had been determined by nuclear magnetic resonance (NMR) analysis [23,24]. This algorithm was selected for multiple sequence alignment of the hPDIR b, hPDI b and b′ domains.

3. Results

3.1. Expression and purification of wild type and domain mutants of hPDIR

To understand the functional roles that are played by the four domains of hPDIR, several hPDIR domain mutant cDNAs were prepared by PCR [25]. The sequences of all cDNAs were confirmed by nucleotide sequence determination (primers not shown). Fig. 1 shows the domain structure of the wild type hPDIR protein and the domain mutants that were used in this study. *E. coli* cells bearing expression plasmids encoding these mutants were cultured as described in Section 2, harvested, disrupted, and the supernatant was applied onto a Ni^{2+}-chelating resin column. The resulting isolated proteins were purified further by using a Hitrap SP FPLC column. All purified proteins appeared as a single band after SDS–PAGE (Fig. 2; the data for hPDIR-b, hPDIR-a^0, hPDIR-a′, hPDIR-ba^{0}a, and hPDIR-a^{0}aa′ have been shown previously [16]).

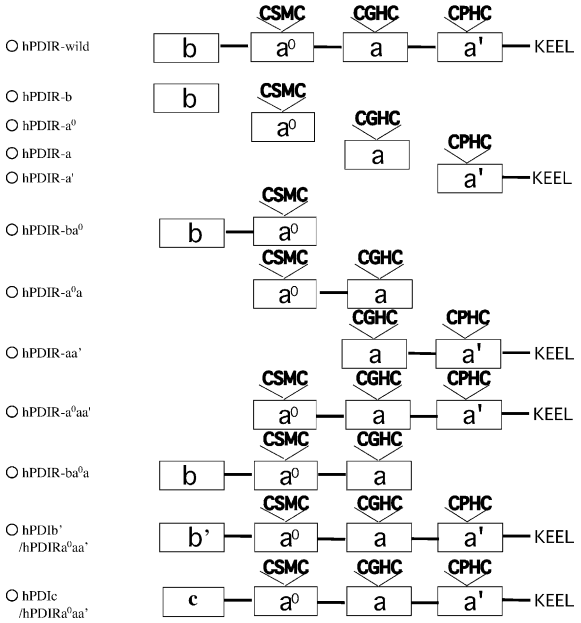

Fig. 1. The domain structure of wild type hPDIR and the hPDIR domain mutants used in this study.

3.2. Isomerase, oxidative refolding, and chaperone activities of wild type hPDIR and domain mutants

As shown in Table 1, the individual domains of hPDIR did not bear significant isomerase activity apart from the a domain (about 48% of the wild-type activity). The domain deletants ba^0, a^{0}a, and aa′ showed about 25%, 78%, and 83% activity, respectively. Notably, these activities were largely equivalent, respectively, to the activities of the hPDIR-m23, hPDIR-m1, and hPDIR m3 mutants that we examined in a previous study [16]. These mutants lack one or two of the three CXXC active sites in hPDIR due to the replacement of the cysteines with

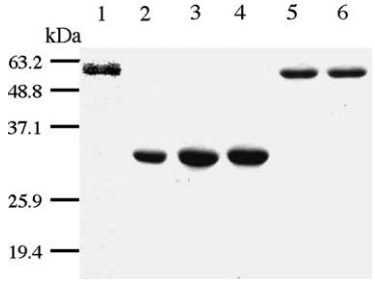

Fig. 2. SDS–PAGE analysis of the hPDIR mutant proteins after their purification by Ni^{2+}-chelating resin column chromatography. Electrophoresis was performed using 15% gels (see Section 2). Lane 1: hPDIR-wild; lane 2: hPDIR-ba^0; lane 3: hPDIR-a^{0}a; lane 4: hPDR-aa′; lane 5: hPDIb′/hPDIRa^{0}aa′; lane 6: hPDIc/hPDIRa^{0}aa′. The numbers to the left indicate the sizes of the molecular weight markers.

Table 1
Activities of isomerase, chaperone, and oxidative refolding of wild and mutant hPDIR proteins

Protein	CXXC motif	% of wild hPDIR activity		
		Isomerase activity	Oxidative refolding	Chaperone activity
hPDm-wild	–CSMC–CGHC–CPHC–	100.00	100.00	100.00
b		N.D.	21.97	43.89
a^0	–CSMC–	9.17	1.09	N.D.
a	–CGHC–	47.72	1.66	N.D.
a′	–CPHC–	11.36	1.67	N.D.
ba^0	–CSMC–	25.14	81.29	50.00
m23	–CSMC–SGHS–SPHS–	24.35	89.73	101.33
a^0a	–CSMC–CGHC–	78.15	1.81	N.D.
m3	–CSMC–CGHC–SPHS–	68.83	93.94	101.16
aa′	–CGHC–CPHC–	83.25	2.42	N.D.
m1	–SSMS–CGHC–CPHC–	82.23	67.18	100.11
a^0aa'	–CSMC–CGHC–CPHC–	96.82	9.68	N.D.
ba^0a	–CSMC–CGHC–	65.92	93.05	98.84
hPDIb′/hPDIRa^0aa'	–CSMC–CGHC–CPHC–	73.18	10.08	186.24
hPDIc/hPDIRa^0aa'	–CSMC–CGHC–CPHC–	101.03	9.67	155.54

The chaperone activity was examined using rhodanese as a substrate. The value of the activity of the wild type hPDIR was set to 100%. Underlined residues indicate cysteine residues that were replaced with serine as described previously [16]. Values for the mutants m23, m3, and m1 were derived from previous data [16]. Each value is shown as the mean of two separate experiments. N.D. indicates not detected.

serine as shown in Table 1. Thus, the m1 and m3 mutants have an inactivated SXXS in their a^0 or a′ domain, respectively, while the a and a′ domains in the m23 mutant both bear an inactivated SXXS. These observations indicate that the presence of only one domain bearing a CXXC motif cannot fully exert the isomerase activity of the protein and that at least two CXXC-bearing domains are required for this activity.

With regard to the ability of hPDIR to oxidatively refold α1-antitrypsin and to serve as a chaperone, the domain deletants a^0, a, a′, a^0a, and aa′ were almost completely inactive (Table 1). In contrast, the oxidative refolding activities of the domain deletants b, ba^0, and ba^0a were about 22%, 81%, and 93% of the wild type activity, while their chaperone activities were about 44%, 50%, and 99%, respectively (Table 1). These observations are consistent with our previous report that showed that the hPDIR mutant that lacks the b domain has poor peptide-binding, chaperone, and α1-antitrypsin oxidative refolding abilities [16]. These observations together indicate that the b domain of hPDIR is critical for binding to its substrates and exerting its chaperone activity, while the a^0, a, and a′ domains (at least two of the three) are needed for isomerase activity.

3.3. Isomerase, oxidative refolding, and chaperone activities of hPDIR mutants whose b domain has been replaced with the b′ or c domains of hPDI

The isomerase activity of hPDIR is much lower than that of hPDI, while its chaperone activity is about 50% of that of hPDI [16]. To determine the basis of these activity differences between hPDI and hPDIR, we constructed hPDIR/hPDI domain chimeras in which the b domain of hPDIR has been replaced with the b′ or c domains of hPDI. These mutants were designated hPDIb′/hPDIa^0aa' and hPDIc/hPDIRa^0aa', respectively. Interestingly, hPDIb′/hPDIa^0aa' showed a dramatic increase in chaperone activity compared to wild type hPDIR (about 186%), while hPDIc/hPDIRa^0aa' also showed an increased activity (about 156%) (Table 1). Their isomerase activities did not increase similarly, as they were about 73% and 101% of that of wild type hPDIR, respectively (Table 1). However, their α1-antitrypsin oxidative refolding abilities were much lower than that of wild type hPDIR (about 10% and 9%, respectively) (Table 1). BIACORE analysis showed that hPDIb′/hPDIRa^0aa' and hPDIc/hPDIRa^0aa' can interact with mastoparan with K_D values of 5.81×10^{-5} and 5.51×10^{-5} M, respectively (data not shown). These values are almost equivalent to those of wild-type hPDI (3.46×10^{-5} M) [16,18] and hPDIR (4.67×10^{-5}M) [16]. These observations indicate that the b domain of hPDIR is critical for its substrate specificity, particularly for the oxidative refolding of α1-antitrypsin, and that the role of the b′ and c domains of hPDI is definite for the chaperone activity.

3.4. Intrinsic fluorescence emission spectra of hPDIR, hPDI, hPDIb′/hPDIRa^0aa', and hPDIc/hPDIRa^0aa'

The increased chaperone activity of hPDIb′/hPDIRa^0aa' and hPDIc/hPDIRa^0aa' prompted us to analyze these mutants biophysically. As shown in Fig. 3, hPDIb′/hPDIRa^0aa' and hPDIc/hPDIRa^0aa' are well structured. The hPDIR, hPDI, hPDIb′/hPDIRa^0aa', and hPDIc/hPDIRa^0aa' proteins all showed a λ_{max} of 335 nm in the fluorescence spectra. However, their intensities differed (Fig. 3). Interestingly, the hPDIb′/hPDIRa^0aa' and hPDI-wild spectra were almost identical, unlike those of hPDIR and hPDIc/hPDIRa^0aa' (Fig. 3). This indicates that the overall structures of hPDIb′/hPDIRa^0aa' and hPDI resemble each other and that replacing the hPDIR b domain with the hPDI b′ or c domains affects the three-dimensional structure of hPDIR.

3.5. Prediction of the secondary structures of the hPDIR b, hPDI b, and hPDI b′ domains

We subjected the hPDIR b, hPDI b, and hPDI b′ domains to secondary structure prediction and multiple sequence alignment. The hPDIR b domain showed 10.52% and 12.28% amino acid sequence similarity to those of the hPDI b and hPDI b′ domains, respectively. Nevertheless, as shown in Fig. 4, the hPDIR b domain has a similar secondary structure to those of the hPDI b and b′ domains, which consist of an α/β fold with the order of the elements of the secondary structure being β1 (“s” in Fig. 4), α1 (“H”), β2 (s), α2 (H), β3 (s), α3 (H), β4 (s), and α4 (H), although the hPDIR b domain does not

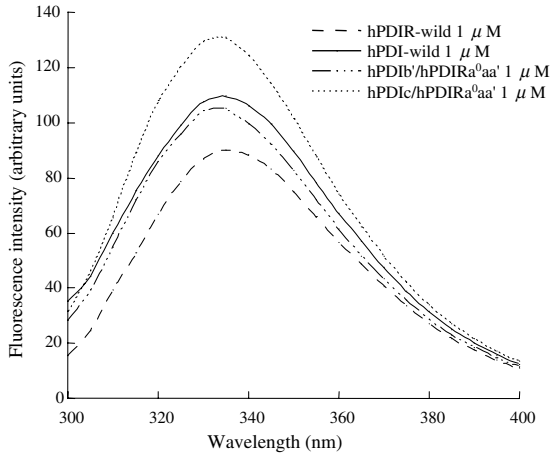

Fig. 3. Intrinsic fluorescence of hPDIR, hPDI, and the hPDIR/hPDI domain chimeras. Shown are the fluorescence spectra of hPDIR, hPDI, hPDIb′/hPDIRa^{0}aa′, and hPDIc/hPDIRa^{0}aa′. All spectra shown are an average of three scans.

have a β5 (s) unlike the hPDI b domain. This suggests that the hPDIR b domain also bears a thioredoxin-like fold, as do the a and b domains of hPDI [23,24].

4. Discussion

We previously reported that hPDIR has isomerase and chaperone activities, although both activities were about 2% and 50% of those of hPDI [16]. We also reported that the three CXXC motifs of hPDIR play different roles in mediating the redox activity of the protein [16]. Although the isomerase activity of hPDIR is much lower than that of hPDI, hPDIR reactivated α1-antitrypsin more effectively compared with hPDI and hP5 [16]. These observations suggest that α1-antitrypsin is one of the specific substrates of hPDIR for catalyzing disulfide bond formation in the ER lumen. In this study, to elucidate the roles played by the individual domains of hPDIR in more detail, we constructed several hPDIR domain deletants. Analysis of these mutants revealed that the domain deletants ba^0, a^{0}a, and aa′, which are constituted of two successive domains, had almost the same respective isomerase activity as the mutants hPDIR-m23, hPDIR-m1, and hPDIR m3 (Table 1). These results indicate that at least two successive domains bearing a CXXC motif are necessary for exerting the isomerase activity of hPDIR. Moreover, while the domain deletants a^0, a, a′, a^{0}a, and aa′ lack chaperone and α1-antitrypsin oxidative refolding activities, the chaperone activities of b, ba^0, and ba^{0}a were about 44%, 50%, and 99% of wild type hPDIR activity, respectively (Table 1). Furthermore, their α1-antitrypsin oxidative refolding activities were about 22%, 81%, and 93%, respectively (Table 1). All single domains b, a^0, a, and a′ were expressed in a soluble form in *E. coli*, and purified to homogeneity. Thus, there was no necessity for refolding. The single domains a^0, a, and a′ exerted isomerase activity, but did not exert oxidative refolding and chaperone activities. On the contrary, the domain b exerted oxidative refolding and chaperone activities, but did not exert isomerase activity. These observations are consistent with our previous report that showed that the hPDIR mutant that lacks the b domain has poor peptide-binding, chaperone, and α1-antitrypsin oxidative refolding activities [16]. Thus, the b domain of hPDIR is critical for its chaperone activity.

While the b domain of hPDIR is needed for both its chaperone activity and its substrate specificity, the chaperone activity of this protein is lower than that of hPDI [16]. To investigate the basis of these differences between hPDI and hPDIR, we constructed the hPDIb′/hPDIa^{0}aa′ and hPDIc/hPDIRa^{0}aa′mutants, since it has been reported that the b′ and c domains of hPDI are critical for its peptide-binding and chaperone activities [26,27]. Interestingly, as shown in Table 1, the chaperone activity of hPDIb′/hPDIRa^{0}aa′ was dramatically higher than that of the wild-type protein (about 186% of wild-type activity), which is almost equivalent to that of hPDI

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 hPDIR b: SAKVSSLIERISDPKDLKKLLRTRNNVLVLYSKSEVAAENHLRLLSTVAQAVKGQGTICWVDCGDAES-RKLCKKMKVDLS
SSTHREAD: ---ssssss-------HHHHHH----sssss------HHHHHHHHHHHHHH-----ssssss-------HHHHHHHHHH-


  hPDI b:  ----PAATTLPDGAAAESLVESSEVAVIGFFKDVES--DSAKQFLQAAEAIDD--IPFGITS--------NSDVFSKYQL
SSTHREAD:  ------------HHHHHHHHH---ssssss--------HHHHHHHHHHHH-----ssssss---------HHHHHHHH-
     NMR:  ------ssss--HHHHHHHHH---sssssss---------HHHHHHHHHH-------sssss---------HHHHHHH-


 hPDI bÕ:  ----PLVIEFTEQTAPKIFGGEIKTHILLFLPKSVSDYDG---KLSNFKTAAESFKGKILFIFIDSDHTDNQRILEFFGL
SSTHREAD:  -----ssssss------------sssssss-----------HHHHHHHH------ssssss--------HHHHHHHH-


 hPDIR b: PKDKKVELFHYQDGAFHTEYNR---AVTFKSIVAFLK
SSTHREAD: ------sssss-------------HHHHHHHH----

  hPDI b: DKDG--VVLFKKFDEGRNNFEGEVTKENLLDFIKHNQL
SSTHREAD: ------sssss-------------HHHHHHHHHH--
     NMR: ----sssssssss----sssss----HHHHHHHHHH--


 hPDI bÕ: KKEECPAVRLITLEEEMTKYKPES-EELTAERITEFCHRFLEGKIKPHLMSQELPEDWDKQP
SSTHREAD: ------ssssss-HHHHH-------HHHHHHHHHHHHHHH-------HHHHHHHHHH-----
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Fig. 4. Secondary structure analysis of the b domain of hPDIR. Of the various protein secondary prediction programs that we tested as described in Section 2, the SSTHREAD algorithm, which employs threading, yielded a secondary structure of the b′ domain of hPDI that fitted the best to the structure that had been determined by NMR [23,24]. This program was used to analyze the secondary structures of the hPDIR and hPDI b′ domains. hPDIR b: human PDIR b domain (2–115); hPDI b: human PDI b domain (118–217); hPDI b′: human PDI b′ domain (218–351). H and s indicate the helix and sheet regions, respectively.

(almost 200% of hPDIR activity). The hPDIc/hPDIRa^{0}aa′ mutant also showed increased chaperone activity (about 156% of wild-type activity) (Table 1). However, the α1-antitrypsin oxidative refolding activities of both chimeric mutants are much lower than that of wild-type hPDIR (Table 1). These results suggest that both the b′ and c domains of hPDI are critical for exerting the chaperone activity and substrate specificity of this protein.

As shown in Fig. 3, the hPDIb′/hPDIRa^{0}aa′ and hPDIc/hPDIRa^{0}aa′ mutants differ structurally from wild-type hPDIR. Interestingly, intrinsic fluorescence spectra showed that hPDIb′/hPDIRa^{0}aa′ structurally resembles wild-type hPDI more than wild-type hPDIR (Fig. 3). This suggests that the structural changes of hPDIR caused by substituting its b domain with the hPDI b′ domain are responsible for its increased chaperone activity. Mastoparan is composed of 14 amino acids and has been reported to bind to hPDI [28] and hPDIR [16]. Despite the structural changes caused by replacing the b domain of hPDIR, BIACORE analysis showed that hPDIb′/hPDIRa^{0}aa′ and hPDIc/hPDIRa^{0}aa′ can interact with mastoparan with K_D values of 5.81×10^{-5} and 5.51×10^{-5} M, respectively (data not shown). These values are almost equivalent to that of wild-type hPDI (3.46×10^{-5} M) [16,18] and hPDIR (4.67×10^{-5} M) [16]. These observations strongly support the notion that the hPDIR b domain and the hPDI b′ and c domains are critical for both the presence and the strength of chaperone activity.

We next subjected the b domain of hPDIR to secondary structure prediction analysis and multiple sequence alignment with the sequences of the hPDI b and b′ domains (Fig. 4). Several programs that predicted secondary structures were tested. The one that yielded a structure that best fitted the structure of the hPDI b domain that was determined by NMR was SSTHREAD [23,24]. This program was selected for the multiple sequence alignment. As shown in Fig. 4, the b domain of hPDIR seems to be structurally similar to the b′ and b domains of hPDI and to bear a thioredoxin-like fold. Kemmink et al. have analyzed the three-dimensional structures of the a and b domains of hPDI by NMR and thereby showed that hPDI consists of active and inactive thioredoxin-like folds [23]. Although the b domain of hPDI is thioredoxin-inactive, the three-dimensional structure of this domain resembles that of the a domain of hPDI, which has a thioredoxin-like fold [23,24]. Moreover, Pirneskoski et al. [29] recently reported that the b′ domain of hPDI also bears a thioredoxin-like fold by using secondary structural prediction and homology modeling. They also reported that the b′ domain of hPDI contains the linker region at its C-terminus, which forms an unaligned extension of the aligned thioredoxin-like section, and designated this region as x region. As shown in Fig. 4, it is suggested that the b domain of hPDIR does not contain the x region. These observations strongly support the notion that, despite the very low level of amino acid sequence similarity of the b domain of hPDIR to the b′ domain of hPDI, this domain also bears a thioredoxin-like fold. However, the b domain of hPDIR does not have a β5 (s) unlike the b domain of hPDI. It is known that the b′ and c domains of hPDI are necessary for the peptide-binding activity of this protein [26,27], and that the b domain of hPDIR determines the substrate specificity of its oxidative refolding activity [16]. This, together with the high level of structural similarity between the hPDIR b domain and the hPDI b′ domain, suggests that these domains are able to discriminate between specific substrates. Pirneskoski et al. [29] also reported that the I^{272} residue in the b′ domain of hPDI is critical for its peptide-binding activity. Our results thus suggest that the b domain of hPDIR may also contain particular amino acids that direct its substrate specificity.

We are currently seeking to identify the critical sites in the b domain of hPDIR that mediates its peptide-binding and substrate specificity by introducing various amino acid mutations.

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