Modulation of cooperativity in *Mycobacterium tuberculosis* NADPH-ferredoxin reductase: Cation- and pH-induced alterations in native conformation and destabilization of the $NADP^+$-binding domain

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Abstract

FprA, a *Mycobacterium tuberculosis* NADPH-ferredoxin reductase, consists of two structural domains, a FAD-binding and a NADP-binding domain, respectively. For the first time, we demonstrated that native FprA, on thermal treatment underwent partial denaturation with unfolding of only the FAD-binding domain and release of the protein-bound flavin. The NADP-binding domain of this protein is highly resistant to denaturation under these conditions. However, the presence of either 150 mM NaCl or KCl or 10 μM $MgCl_2$ or $CaCl_2$ or slightly acidic pH of 6.0 resulted in a highly cooperative and complete thermal unfolding of the protein. Physicochemical investigations showed that the monovalent cations or low concentrations of divalent cations induced compaction of the protein conformation. However, divalent cations at higher concentrations resulted in FAD release leading to stabilization of an enzymatically inactive apo-enzyme. Detailed thermal denaturation studies on the native protein and the isolated NADP-binding domain showed that cations and pH 6.0 destabilized only the heat-stable NADP-binding domain. The experimental studies demonstrate that modulation of intramolecular ionic interactions induce significant conformational changes in the NADP-binding domain of FprA, resulting in a substantial increase in the structural cooperativity of the whole molecule. The results presented in this paper are of importance as they demonstrate alterations in the native three-dimensional structure of FprA and cooperativity in protein molecule on slight alteration of pH or modification of ionic interactions in protein.

Keywords: flavoprotein; *Mycobacterium tuberculosis*; cooperativity; thermal denaturation; cation

Tuberculosis is still a major cause of mortality in both developing and industrialized countries. It kills about three million people every year, and its diffusion is constantly increasing due to development of drug resistance, duration of the therapeutic treatment, and increased susceptibility of immunocompromised individuals. The availability of the genome sequence of its etiological agent, *Mycobacterium tuberculosis* (Cole et al. 1998), allowed to identify new potential targets for developing novel anti-tubercular drugs.

M. tuberculosis grows within phagocytic vacuole of macrophages. The key to success of *M. tuberculosis* as a pathogen depends on its ability to maintain an infection inside the phagocytic vacuole of the macrophage where it encounters an acidic environment. To survive under these conditions, the *M. tuberculosis* has to limit acidification of the phagosome, which it does successfully to some extent by utilizing transport systems which exchange protons for cations re-

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Abbreviations: FprA, NADPH-ferredoxin reductase; T_m, midpoint of thermal denaturation.

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sulting in increased levels of these cations within the cell (Sturgill-Koszcki et al. 1994; Piddington et al. 2000; Pieters 2001). Furthermore, under these conditions the vacuole is moderately acidic with pH of around 6 (Xu et al. 1994). pH and cations modulate the electrostatic interactions among charged moieties of surface ionizable residues present in the proteins resulting in alterations in the conformation and stability of proteins (Ahmad et al. 2001; Maldonado et al. 2002). Due to these facts it can be assumed that the proteins of *M. tuberculosis*, especially acidic proteins, can experience alterations in the native conformation when the organism is present in the phagocytic vacuole.

FprA is a *M. tuberculosis* flavoenzyme encoded by gene Rv3106 of the H37Rv strain of the pathogen (Cole et al. 1998). This is a 50 kDa oxidoreductase that transfers in vitro two reducing equivalents from NADPH to ferredoxins of the 3Fe and 7Fe types, via the protein-bound FAD cofactor (Fischer et al. 2002). Although the exact physiological role of this flavoprotein is still to be established, insights have been derived from its primary structure. FprA shows significant sequence homology with the mammalian adrenodoxin reductase and with its yeast homolog Arh1p (Manzella et al. 1998). This information suggests a possible involvement of FprA as an electron donor in processes such as cytochrome P450 monooxygenase reactions and iron–sulfur cluster biogenesis. Indeed, the *M. tuberculosis* genome contains 20 genes encoding putative cytochrome P450 proteins (Cole et al. 1998), which are probably required for fatty acid and mycolipid oxygenation. The enzyme FprA has thus been suggested to be a potential drug target of *M. tuberculosis*. The atomic resolution structure of FprA in the oxidized and NADPH-reduced forms have been published (Bossi et al. 2002). Although the enzyme is monomeric in solution (Fischer et al. 2002), it crystallized in dimeric form (Bossi et al. 2002). However, the dimer was suggested to be the result of crystal packing. Structurally, the overall architecture of the FprA protein is similar to that observed for proteins belonging to the family of glutathione reductase (Dym and Eisenberg 2001), of which FprA is a member. The FprA monomer consists of two domains, both exhibiting a Rossmann fold topology (Schulz 1992). The FAD-binding domain of the enzyme consists of the N- and C-terminal regions of the protein, whereas the central part of the polypeptide chain constitutes the NADP-binding domain. A small two-stranded β-sheet links the two domains. Recently, a further, more detailed functional characterization of FprA had been reported (McLean et al. 2003), which provided a picture of the thermodynamic and transient-kinetic properties of the enzyme.

We have carried out comparative thermal unfolding studies on FprA in the presence and absence of cations and at slightly acidic pH. Detailed physicochemical studies on the effect of cations, both monovalent and divalent, on the structural and functional properties of FprA were carried out using various optical spectroscopic techniques, like fluorescence and CD. The changes in the molecular dimension of the protein on incubation with cations were studied by size-exclusion chromatography. Identification of the structural domain of FprA whose thermal stability is modulated by cations was also pursued by limited proteolysis.

Results

The crystal structure of FprA shows that the enzyme is made of two structural domains connected by a short two-stranded β-sheet linker (Bossi et al. 2002). The FAD-binding domain consists of residues 2–106 from the N-terminal region and residues 327–456 from the C-terminal region. The NADP-binding domain consists of residues 110–323 from the central part of the polypeptide. The two structural domains of FprA are linked by the residues 107–109 and 324–326, which form the two-stranded anti-parallel β-sheet.

Lack of cooperativity in native FprA molecule

To analyze whether the two structural domains of FprA interact strongly with each other or they are independent folding/unfolding units, we carried out thermal denaturation experiments by monitoring the loss of secondary structure of the enzyme at increasing temperatures. In the far-UV region, the CD spectrum of native FprA shows the presence of a substantial amount of α-helical conformation (McLean et al. 2003). Hence, loss of CD signal at 222 nm was taken as a measure of the unfolding of FprA. Figure 1 shows the

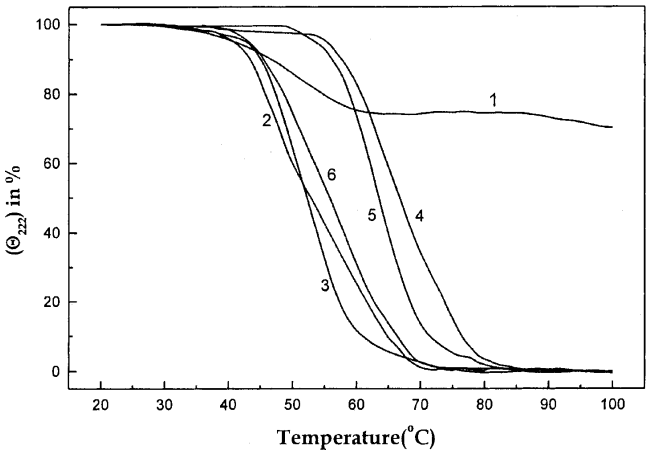

Figure 1. Effect of cations on the thermal denaturation of FprA. Thermal denaturation profiles of FprA in the absence and in the presence of different cations, as measured by loss of CD ellipticity at 222 nm. The curves 1 to 6 represent samples of FprA at low ionic strength and in the presence of NaCl, KCl, $MgCl_2$, $CaCl_2$, and pH 6.0, respectively. When present, monovalent cations were at the concentration of 150 mM and the divalent cations at 10 μM. The CD signal loss is represented as percentage of the value observed for the protein sample at 20°C.

changes in CD ellipticity at 222 nm of FprA as a function of temperature. A broad sigmoidal transition between 30°C to 65°C having an apparent T_m (midpoint of thermal denaturation) of about 49°C was observed. As the temperature was increased above 80°C, a further slight decrease in the CD signal at 222 nm was seen. An interesting observation was that a loss of only about 27% CD ellipticity at 222 nm was associated with the thermal denaturation of FprA, demonstrating that the major part of the protein molecule is resistant to thermal unfolding. This indicates that FprA is composed of two unfolding units that behave independently and have different thermal stabilities under low ionic strength conditions: one is sensitive to thermal denaturation for which a transition is observed at 49°C, while the other is stable at temperatures up to 80°C. Hence, native FprA is a noncooperative molecule in which two distinct structural units fold/unfold independently from each other.

Cations and pH 6.0 induced cooperativity in FprA

The effect of cations on the thermal denaturation of the FprA molecule was studied by incubating the protein with salts like NaCl, KCl, $MgCl_2$, and $CaCl_2$. As all these salts contain the same anion (i.e., Cl^-), the different effects observed in the comparative study using these salts will be mainly due to the different cations. Figure 1 summarizes the thermal denaturation profiles of NaCl-, KCl-, $MgCl_2$-, $CaCl_2$-, and pH 6.0-incubated FprA, as monitored by loss of CD ellipticity at 222 nm. All these samples showed a single sharp sigmoidal transition with almost complete loss of secondary structure associated with the transition. However, differences were observed in the T_m associated with the transitions under these conditions. These observations demonstrated that, unlike native FprA, which on thermal denaturation is only partially unfolded, the cation- or pH 6.0-incubated FprA undergoes a simultaneous unfolding of both the domain on thermal treatment, probably as a result of cooperativity induced in the enzyme under these conditions.

Effect of NaCl on the structural and functional properties of FprA

Time-dependent changes in the structural parameters and enzymatic activity of FprA at increasing salt concentrations were monitored to standardize the incubation time required for achieving equilibrium under each condition. At 0.15, 0.5, and 1 M NaCl, changes in tryptophan and FAD fluorescence were complete within 30 min, with no further variation observed in the next 12 h (data not shown). These observations indicate that an incubation of about 1 h is sufficient for achieving equilibrium under any salt condition tested.

Figure 2 summarizes the effect of increasing concentration of NaCl on the tertiary and secondary structure of FprA as studied by monitoring the changes in the tryptophan and FAD fluorescence, and CD ellipticity at 222 nm, respectively. Figure 2A reports the effect of the salt on the tryptophan fluorescence of the protein. A decrease of the wavelength of the tryptophan emission maximum from 338 nm to 334 nm was observed by increasing the NaCl concentration from 0 M to 0.5 M. This suggests that incubation of FprA with NaCl leads to movement of tryptophan residues present in the protein to a more hydrophobic environment, probably as a result of compaction of native conformation of the protein molecule (Lakowicz 1983; McLean et al. 2003). However, no significant changes in fluorescence polarization (Fig. 2B) and enzymatic activity (Fig. 2C) of FprA on incubation with increasing NaCl concentrations was observed. As far as the secondary structure of FprA is concerned, only a small enhancement, about 6%, was observed as the salt concentration was increased from 0 M to 0.5 M NaCl (Fig. 2D). These observations collectively suggest that the incubation of FprA with NaCl resulted in a compaction of the conformation of the protein without large alteration in its secondary structure, FAD microenvironment, or functional activity. This conclusion was further supported by size-exclusion chromatography in the presence and in the absence of salts. In Figure 2E the chromatograms of FprA obtained at different NaCl concentrations are reported. In the absence of NaCl, FprA eluted as a single symmetric peak with a retention volume of 15.0 mL, corresponding to the expected molecular mass of about 50 kDa. However, in the presence of either 0.15 or 0.5 M NaCl, the elution volume for FprA was increased to 15.2 and 15.5 mL, respectively. This indicates a significant reduction of the hydrodynamic radius for the NaCl-stabilized conformation of FprA, compared to the conformation adopted by the native protein, thus confirming that incubation of FprA with NaCl results in compaction of its conformation.

Effect of $CaCl_2$

Figure 3A shows the effect of increasing concentrations of $CaCl_2$ on the tryptophan fluorescence. An initial sharp decrease in the wavelength of the emission maximum from 338 nm to 334 nm was observed as $CaCl_2$ concentration was increased from 0 M to 0.1 M. A further increase in the $CaCl_2$ concentration up to 1 M reverted this effect, bringing the wavelength of the emission maximum to 337 nm. This suggests that the FprA molecule undergoes a compaction in conformation in the presence of low (up to 0.1 M) $CaCl_2$ concentrations. In this respect, the effect of Ca^{2+} at low concentrations is similar to that of Na^+. This is further supported by the studies with 1 M NaCl-stabilized FprA as shown in Figure 3A. No significant change in tryptophan fluorescence emission maxima of 1 M NaCl-stabilized FprA was observed up to 0.4 M $CaCl_2$ concentration, suggesting

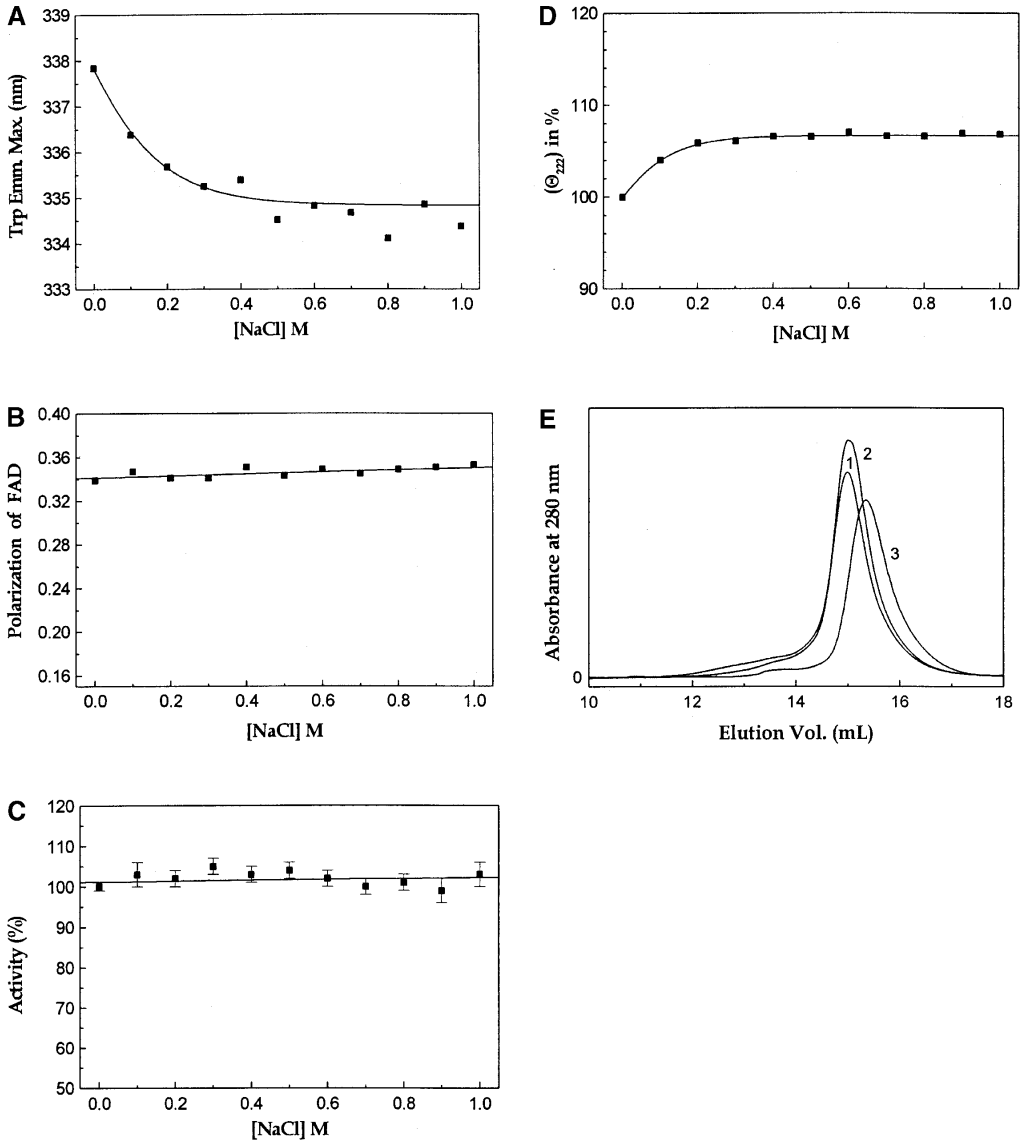

Figure 2. Changes in the structural properties of FprA on incubation with increasing concentrations of NaCl at pH 7.0 and 25°C. (*A*) Changes in the wavelength of the tryptophan fluorescence emission maximum of FprA with increasing concentrations of NaCl. (*B*) Changes in the FAD fluorescence polarization of FprA with increasing concentrations of NaCl. (*C*) Changes in the enzymatic activity of FprA with increasing concentrations of NaCl. The values are represented as percentage of the value observed for FprA in the absence of NaCl. (*D*) Changes in the CD ellipticity at 222 nm of FprA with increasing concentrations of NaCl. The values are represented as percentage of the value observed for FprA in the absence of NaCl. (*E*) Size-exclusion chromatographic profiles of FprA at increasing concentrations of NaCl on a Superdex 200HR column at pH 7.0 and 25°C. The curves 1 to 3 represent the profiles of FprA at low ionic strength and in the presence of 0.15 M and 0.5 M NaCl, respectively.

that the NaCl-stabilized compact conformation of enzyme (as discussed above) is not affected by $CaCl_2$. However, at variance with the case of the monovalent cation, a further increase in $CaCl_2$ concentration (from 0.1 M to 1 M and 0.4 M to 1 M for native and 1 M NaCl-stabilized FprA, respectively) led to a relaxation of the $CaCl_2$-stabilized compact conformation of FprA.

The spectral characteristics of the prosthetic group FAD in flavoproteins have been demonstrated to be sensitive to the protein microenvironment (Ghisla et al. 1974; Visser et al. 1995). FprA contains a tightly but noncovalently bound FAD molecule, buried in the molecule interior. Fluorescence of the FprA-bound FAD is nearly completely quenched, with a high polarization of the residual emission. As shown in Figure 3B, incubation of FprA with increasing concentration of $CaCl_2$ in the range of 0.1 M to 0.8 M, resulted in a significantly large sigmoidal decrease in FAD fluorescence polarization from 0.33 to 0.13. This observa-

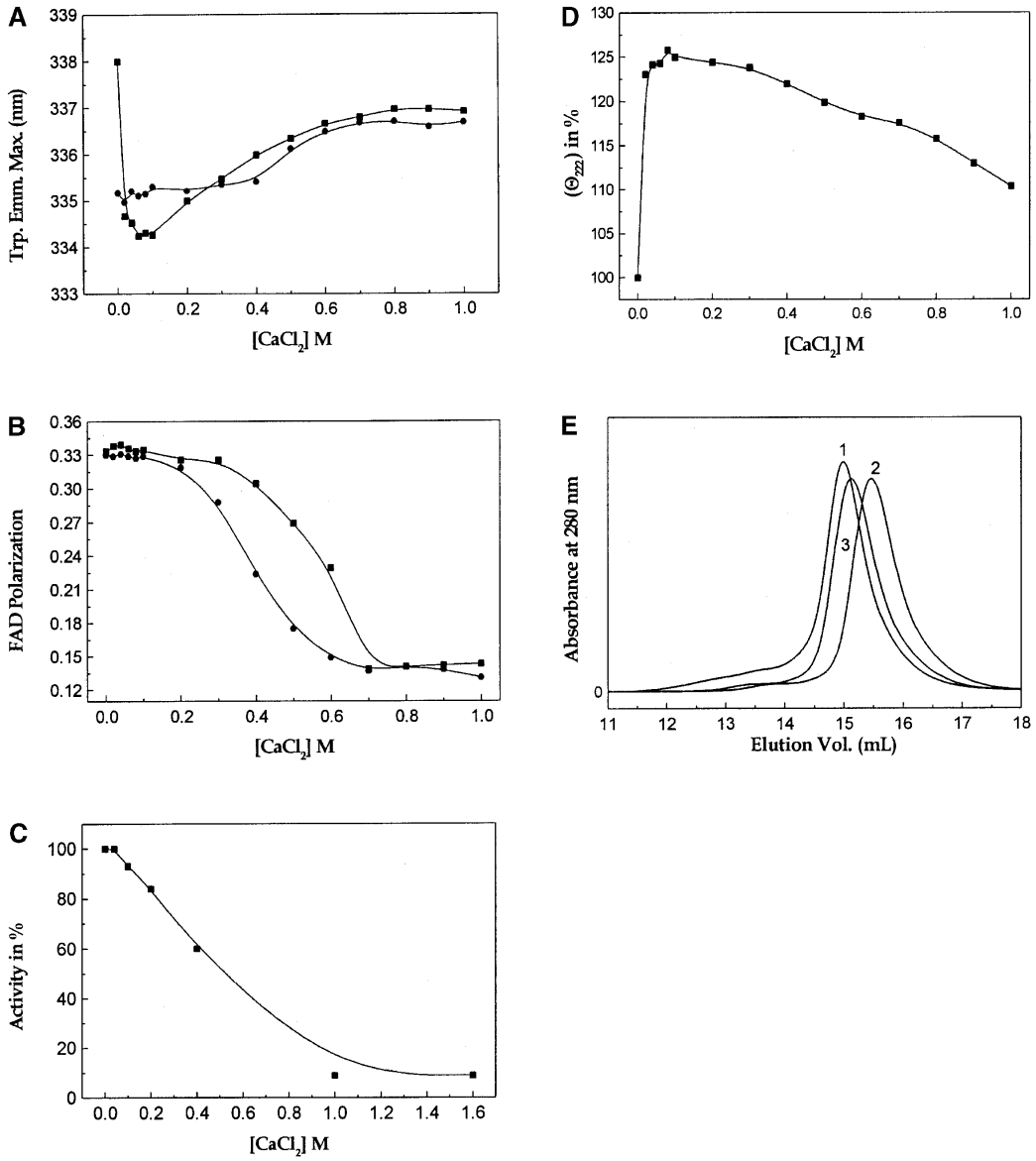

Figure 3. Changes in the structural properties of FprA on incubation with increasing concentrations of $CaCl_2$ at pH 7.0 and 25°C. (*A*) Changes in the wavelength of the tryptophan fluorescence emission maximum of FprA with increasing concentrations of $CaCl_2$. The squares represent data with native protein and the circles that with 1 M NaCl-stabilized protein. (*B*) Changes in the FAD fluorescence polarization of FprA with increasing concentrations of $CaCl_2$. The symbols are same as in *A*. (*C*) Changes in the enzymatic activity of FprA with increasing concentrations of $CaCl_2$. The values are represented as percentage of the value observed for FprA in the absence of $CaCl_2$. (*D*) Changes in the CD ellipticity at 222 nm of FprA with increasing concentrations of $CaCl_2$. The values are represented as percentage of the value observed for FprA in absence of $CaCl_2$. (*E*) Size-exclusion chromatographic profiles of FprA at increasing concentrations of $CaCl_2$ on a Superdex 200HR column at pH 7.0 and 25°C. The curves 1 to 3 represent profiles of FprA at low ionic strength and in the presence of 0.1 M and 1.0 M $CaCl_2$, respectively.

tion suggests that incubation of FprA with high concentrations of $CaCl_2$ resulted in alterations of the conformation of protein such that the protein-bound FAD became either solvent exposed or dissociated from the protein molecule. To assess whether $CaCl_2$ induced the release of FAD from the apoprotein, studies as those reported earlier were undertaken (Ahmad et al. 2001). After incubation with 1 M $CaCl_2$, FprA was concentrated on a Centricon ultrafiltration device with a 3 kDa cutoff. The presence of FAD in free form (in filtrate) and protein-bound form (in protein fraction) was monitored by fluorescence spectroscopy. Under these conditions, a major fraction of FAD was observed in the filtrate (~80% relative fluorescence) with a little fraction associated with the protein (~16% relative fluorescence). In

the absence of $CaCl_2$, under the same treatment, most of the FAD (~90%) remained protein-bound to FprA. This conclusion was confirmed by following the enzymatic activity of FprA: a decrease of activity was observed as the $CaCl_2$ concentration was increased from 0 M to 1 M (Fig. 3C), with only 9% residual activity at 1 M $CaCl_2$. The $CaCl_2$-induced release of FAD from FprA seems probably due to specific interaction of Ca^{2+} ions with the enzyme. This possibility is supported by the observation that for 1 M NaCl-stabilized enzyme the release of FAD from the enzyme occurs at a significantly lower $CaCl_2$ concentration than that observed for the native protein (Fig. 3B). These observations demonstrate that incubation of FprA with $CaCl_2$ leads to dissociation of protein-bound FAD from the protein molecule as a result of specific interaction of Ca^{2+} ions with protein. Figure 3D shows the changes in the secondary structure of FprA on incubation with increasing $CaCl_2$ concentration as monitored by changes in CD ellipticity at 222 nm. With increasing salt concentration, an initial sharp enhancement of about 25% in ellipticity was observed. This was followed by a smoother and continuous decrease in ellipticity with further increase in $CaCl_2$ concentration up to 1 M. However, even at 1 M $CaCl_2$, the CD ellipticity value at 222 nm was slightly greater than that observed for the FprA holoprotein in the absence of Ca^{2+}. These observations suggest that incubation of FprA with low concentrations of $CaCl_2$ induced an increase of the secondary structure content of protein, but that further increase in $CaCl_2$ above 0.1 M concentration resulted in relaxation of the $CaCl_2$-induced secondary structure, along with release of the bound flavin. However, the fact that the 1 M $CaCl_2$-stabilized FprA conformation had a secondary structure content slightly higher than that observed in the absence of Ca^{2+} suggests that the $CaCl_2$-stabilized apoprotein of FprA also adopts a compact conformation.

These conclusions were supported by comparative size-exclusion chromatography studies on FprA in the absence and in the presence of different $CaCl_2$ concentrations. Figure 3E summarizes the results carried out on a S-200 Superdex column. When FprA incubated with either 0.1 or 1 M $CaCl_2$ was chromatographed on the column in the presence of the same $CaCl_2$ concentrations in the mobile phase, significant increases in the retention volumes to 15.5 and 15.2 mL, respectively, were observed, compared to the value 15.0 mL obtained for FprA in the absence of salts. This increase in retention volume is indicative of a significantly reduced hydrodynamic radius of the $CaCl_2$-stabilized forms of FprA compared to the native protein. Thus, $CaCl_2$ induced a compaction in the conformation of both the apoprotein and the holoprotein forms of FprA. Furthermore, the conformation of the FprA form stabilized at low $CaCl_2$ concentration (0.1 M) shows a higher degree of compaction in comparison to that of the apoprotein form stabilized at higher $CaCl_2$ concentration.

Insights in the mechanism of the cation- or pH-induced cooperativity in the thermal unfolding of FprA

On thermal denaturation, the FprA molecule was found to undergo only partial unfolding. However, in the presence of monovalent or divalent cations a complete and cooperative thermal unfolding process was observed. To understand the underlying mechanism of cation-induced cooperativity in FprA, we carried out denaturation studies on protein incubated with increasing concentration of salts. Figure 4, A and B, summarizes the thermal unfolding profile of FprA in the presence of increasing concentration of KCl and NaCl, respectively, as monitored by the loss of the CD ellipticity at 222 nm. For FprA incubated with low concentrations of salts (20 or 30 mM), a profile including a first transition followed by a further loss of the CD signal occurring at much higher temperatures, was obtained. The first transition was in the temperature range of 35°C to 65°C, with only about 30% loss of secondary structure, similar to what was observed in the thermal denaturation of FprA in absence of salts, whereas the second process occurred at temperatures above 85°C and 75°C in the presence of 20 mM and 30 mM KCl, respectively, and resulted in loss of about 80% of the CD signal at 100°C. As the KCl concentration was increased to 40 mM, two distinct transitions were observed, showing T_m values of about 49°C and 58°C, respectively. These observations demonstrate that NaCl or KCl affected only the T_m of the second transition, whereas the first transition remained unchanged as the salt concentration was increased. At KCl or NaCl concentrations of about 150 mM, only a single transition with a T_m of about 52°C was observed, resulting to the complete loss of the secondary structure of the protein. Further increase in KCl or NaCl concentration upto 1 M did not show any significant change in the thermal denaturation profile or T_m. The effect of $MgCl_2$ and $CaCl_2$ on the thermal denaturation of FprA was evident at much lower concentrations (Fig. 4C,D). At a $MgCl_2$ and $CaCl_2$ concentration of 1 μM, respectively, two distinct transitions were observed. This pattern switched to a monophasic profile with complete unfolding of FprA at $MgCl_2$ and $CaCl_2$ concentrations of 10 μM. Furthermore, unlike the NaCl or KCl, the $MgCl_2$ or $CaCl_2$ showed an interesting observation. With increasing concentration of $MgCl_2$ and $CaCl_2$ a significant decrease in T_m associated with the thermal unfolding was observed. These results demonstrate that, in comparison to NaCl or KCl, $MgCl_2$ and $CaCl_2$ were more effective in destabilizing the heat-resistant structural unit of FprA. Furthermore, higher concentrations of $CaCl_2$ and $MgCl_2$ significantly affected the protein structure, resulting in some degree of destabilization of the whole protein molecule.

The studies described so far suggest that modulation of electrostatic interactions within the heat-resistant structural unit of FprA by cations leads to its destabilization and in-

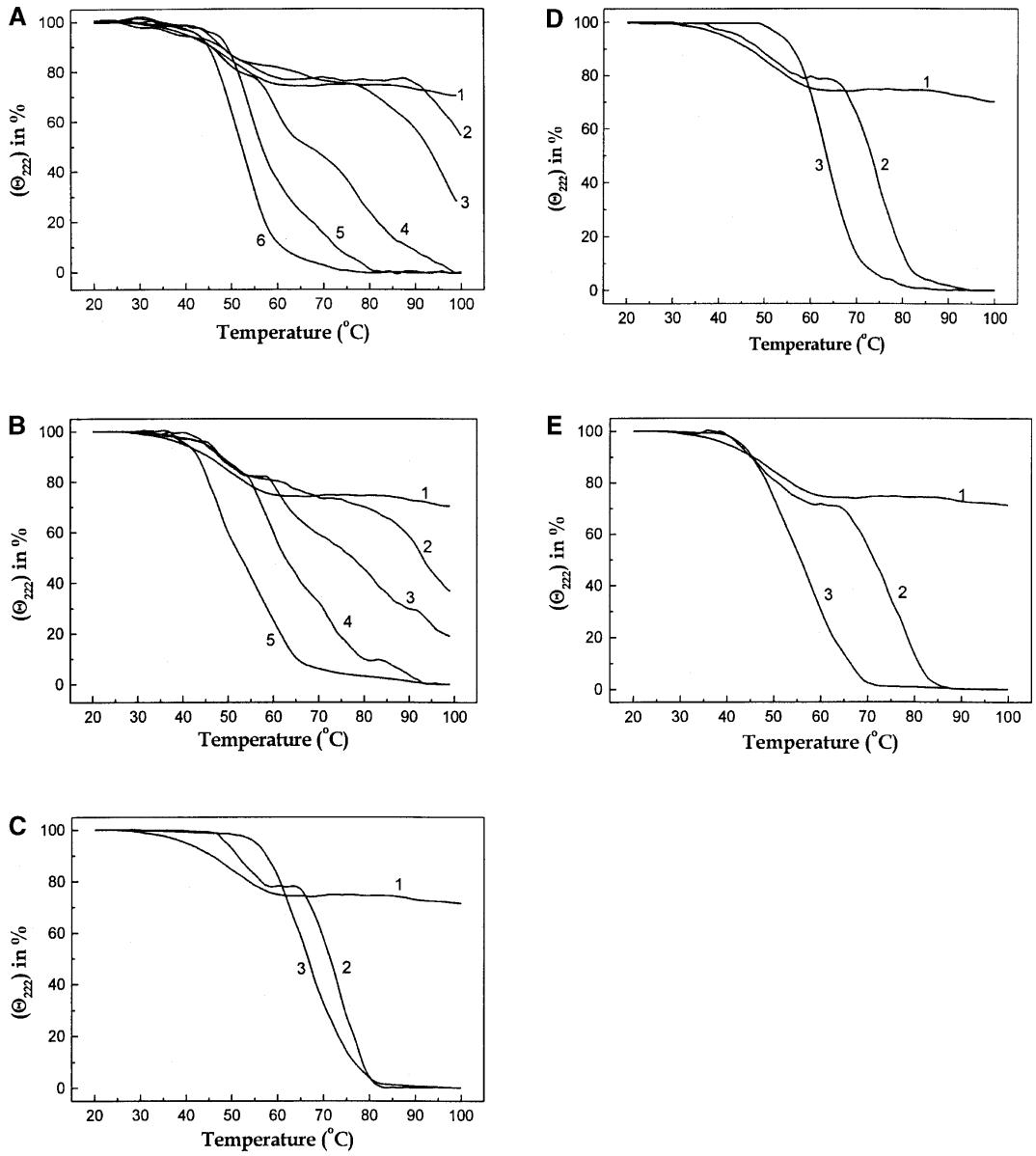

Figure 4. Thermal denaturation profiles of FprA in the presence of increasing concentrations of cations, and at acidic pH, as measured by loss of CD ellipticity at 222 nm. (*A*) Thermal denaturation profiles of FprA incubated with increasing concentrations of KCl. The curves 1 to 6 represent profiles for FprA at low ionic strength, and in the presence of 20, 30, 40, 60, and 150 mM of KCl, respectively. (*B*) Thermal denaturation profiles of FprA incubated with increasing concentrations of NaCl. The curves 1 to 5 represent samples of FprA at low ionic strength, and in the presence of 30, 40, 60, and 150 mM of NaCl, respectively. (*C*) Thermal denaturation profiles of FprA incubated with increasing concentrations of $MgCl_2$. The curves 1 to 3 represent samples of FprA at low ionic strength, and in the presence of 1 and 10 μM $MgCl_2$, respectively. (*D*) Thermal denaturation profiles of FprA incubated with increasing concentrations of $CaCl_2$. The curves 1 to 3 represent samples of FprA at low ionic strength, and in the presence of 1 and 10 μM $CaCl_2$, respectively. (*E*) Thermal denaturation profiles of FprA between pH 7.0 and 6.0 as measured by loss of CD ellipticity at 222 nm. The curves 1 to 3 represent the denaturation of FprA at pH 7.0, 6.4, and 6.0, respectively. In all the figures the values for loss of CD signal are represented as percentage of the value observed for the protein sample at 20°C.

duces cooperativity in the otherwise structurally noncooperative FprA molecule. An obvious way to affect charge-to-charge interactions in proteins is to change the protonation of ionizable group by changing the pH of the medium. Thus, to further support our conclusions about the role of ionic interactions in FprA, we carried out a survey on the effect of pH on the thermal denaturation of the enzyme. Figure 4E reports the thermal unfolding profiles of FprA at different pH values within the range of 6.0–7.0, as monitored by the loss of CD ellipticity at 222 nm. As the pH was

lowered from pH 7.0 to 6.4, the unfolding of the heat-resistant structural unit of FprA was obtained with increasingly lower value of T_m. The profiles were clearly biphasic under these conditions, indicating lack of cooperativity in enzyme molecule. However, lowering the pH to 6.0 resulted in a further destabilization of the heat-resistant unit. Under these conditions, the unfolding profile of FprA showed a single transition with a T_m of about 56°C, corresponding to a cooperative and complete loss of the secondary structure of the protein.

pH-dependent changes in structure and function of FprA were also studied. Figure 5 shows the changes in tryptophan florescence emission maxima, FAD fluorescence, enzymatic activity, and CD ellipticity at 222 nm of FprA in the pH range of 7.0 to 4.0. A significant loss of secondary structure and enzymatic activity of FprA as well as a significant

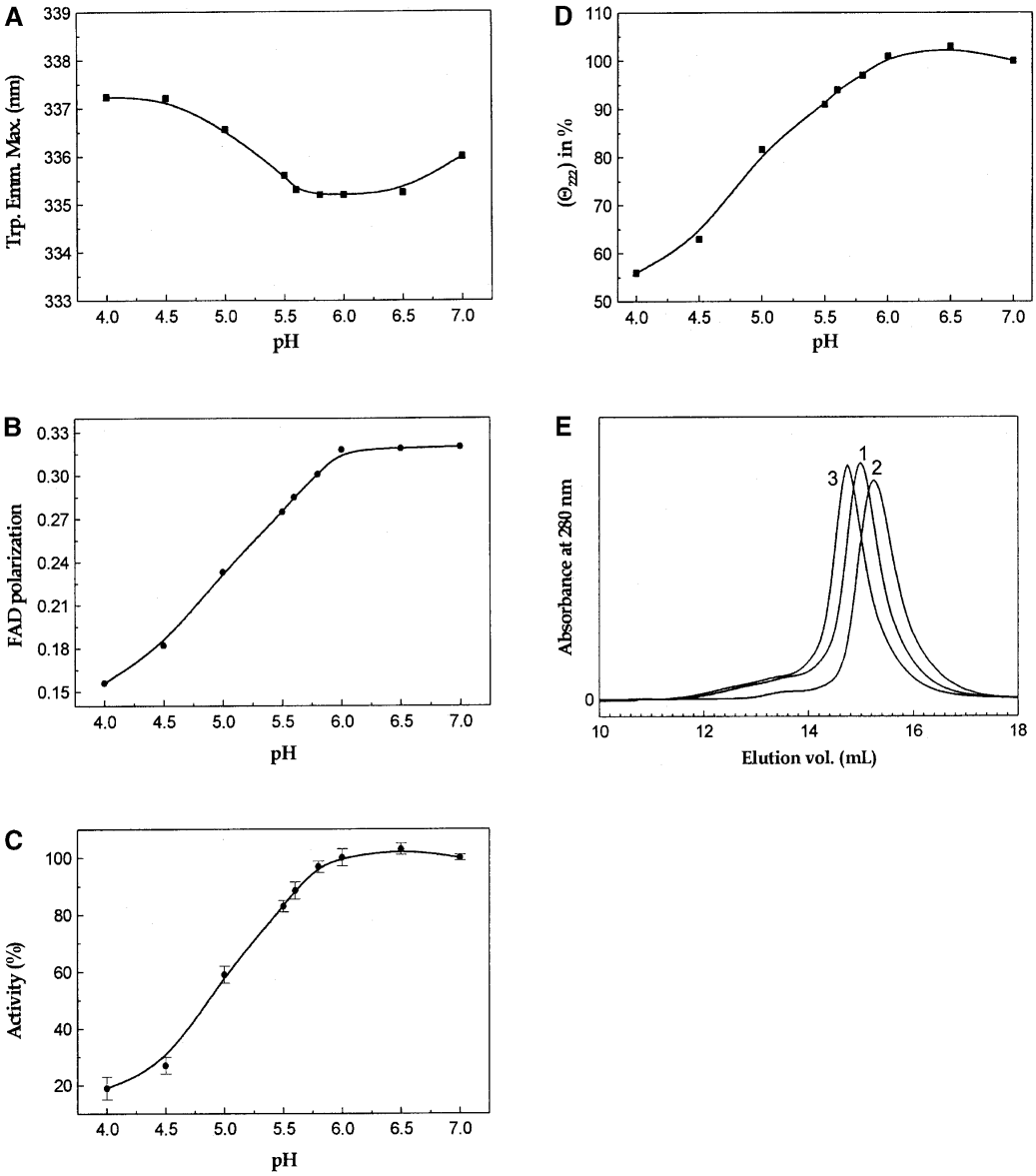

Figure 5. pH-Induced changes in the structural properties of FprA between pH 7.0 and 4.0 at pH 7.0 and 25°C. (*A*) Changes in the wavelength of the tryptophan fluorescence emission maximum of FprA with decreasing pH. (*B*) Changes in the FAD fluorescence polarization of FprA with decreasing pH. (*C*) Changes in the enzymatic activity of FprA with decreasing pH. The values are represented as percentage of the value observed for FprA at pH 7.0. (*D*) Changes in the CD ellipticity at 222 nm of FprA with decreasing pH. The values are represented as percentage of the value observed for FprA at pH 7.0. (*E*) Size-exclusion chromatographic profiles of FprA at decreasing pH on a Superdex 200HR column at 25°C. The curves 1 to 3 represent the profiles of FprA at pH 7.0, 6.0, and 5.0, respectively.

exposure of the buried tryptophan and FAD molecule present in the native conformation was observed on decrease in pH from 6.0 to 4.0. These observations suggest a significant unfolding of FprA under these conditions. Between pH 7.0 and 6.0 no significant changes in enzymatic activity or secondary structure of protein was observed. However, a shift in fluorescence emission maxima of tryptophan fluorescence from 337 nm to 335 nm was observed on changing the pH from 7.0 to 6.0, which indicates the possibility of compaction of conformation of FprA under these conditions (as discussed earlier). This possibility was confirmed by SEC studies (Fig. 5E), where a significant enhancement in retention volume (retention volume ~15.21 mL at pH 6.0) compared to native protein (retention volume ~15 mL at pH 7.0) was observed for protein at pH 6.0. For protein at pH 5.0 (retention volume ~14.75 mL) a partial unfolding of protein is observed.

Identification of the structural unit destabilized by cations

As FprA contains two structural domains, the FAD-binding domain and the NADP-binding domain, we tried to identify which of these domains was the heat-resistant unit of the protein. We first studied the effect of thermal denaturation on the protein bound FAD molecule in FprA. Figure 6A shows the effect of thermal denaturation on the FAD environment of FprA, as studied by loss of the CD signal at 370 nm, which is a measure of the release of the FAD to a nonchiral environment (Maeda et al. 2002; McLean et al. 2003). A single sigmoidal transition between 43°C and 80°C having a T_m of about 56°C was observed. The loss of the CD ellipticity at 370 nm demonstrates that thermal denaturation of FprA led to the release of the protein-bound FAD from the protein molecule. The temperature at which the CD signal corresponding to FAD dissociation started decreasing (~47°C) is close to the T_m of 49°C observed for the loss of secondary structure (Fig. 1A). This observation suggests that during the thermal denaturation of FprA the unfolding of the heat-sensitive unit occurs first, followed by the release of FAD from the partially unfolded protein. Thus, the heat-sensitive unit of the protein corresponds to the FAD binding domain, and hence, the NADP-binding domain of FprA should be the heat-resistant domain. To unequivocally prove this we used a limited proteolysis approach. The factors determining the vulnerability to proteolysis of a protein depends on conformational parameters such as accessibility, segmental motion, and protrusion. For this reason, limited proteolysis has been effectively used to identify structural domains in proteins, ligand-induced conformational changes, and to monitor protein folding and unfolding processes (Hubbard 1998). Figure 6B shows the SDS-PAGE profile of the protein digest obtained by limited proteolysis of FprA with α-chymotrypsin at 45°C, conditions under which the heat-sensitive unit should be mostly unfolded. Besides several minor bands, a major protein band, with a molecular mass of about 24 kDa, was observed. This 24 kDa protease-resistant FprA fragment showed high affinity to Cibacron Blue resin (Fig. 6C). Indeed, it eluted at a higher salt concentration in comparison to the native FprA, indicating that the protease-resistant fragment binds more strongly to the Cibacron Blue resin than the native protein. The nature of the interaction of the proteolysed FprA with the resin ligand was further investigated by examining the effect of presence of $NADP^+$ in the sample. An earlier elution of the protease-resistant fragment was obtained when 0.5 mM $NADP^+$ was included in the mobile phase, demonstrating that $NADP^+$ was able to avoid binding of the protein to the resin, although only partially. The above observations indicate that the intact protein fragment obtained on proteolysis of FprA with α-chymotrypsin is probably the NADP-binding domain of protein. This was confirmed by N-terminal sequencing of the protein fragment obtained on proteolysis of FprA with α-chymotrypsin. The first five amino acids of the protein fragment were VGWYN, suggesting that the protease acted between the amino acid residues Phenyalanine 128 and Valine 129 in the primary sequence of FprA, and as a result, the NADP-binding domain (amino acid 129–334) is obtained.

The NADP-binding domain of FprA obtained by limited proteolysis was purified by affinity chromatography on a Cibacron Blue column. In Figure 6D the size-exclusion chromatography profile of the purified domain is compared to that of native FprA. The purified preparation of the FprA fragment yielded a single peak with a molecular weight of about 25 kDa. These results were confirmed by electrospray mass spectrometry (data not shown). The effect of NaCl and pH on the thermal denaturation of the isolated NADP-domain of the protein was then studied. On thermal denaturation, the FprA fragment showed a single transition, occurring in the temperature range of 50°C to 80°C, with an apparent T_m of about 62°C, and corresponding to a loss in secondary structure of about 15% (Fig. 5E). When the proteolytic fragment was incubated at pH 6.0 or with 150 mM NaCl at pH 7.0 and then subjected to thermal denaturation, a single sigmoidal transition, however, with significantly decreased T_m of 52°C and 50°C, respectively, and a complete loss of the secondary structure was obtained. These observations demonstrate that the isolated $NADP^+$-binding domain of FprA is indeed resistant to thermal denaturation, and interaction of this domain with NaCl leads to its significant destabilization.

Discussion

Based on the results presented in this paper, the effect of modulation of the ionic interactions in FprA by cations on the cooperativity, structural, and functional properties of the

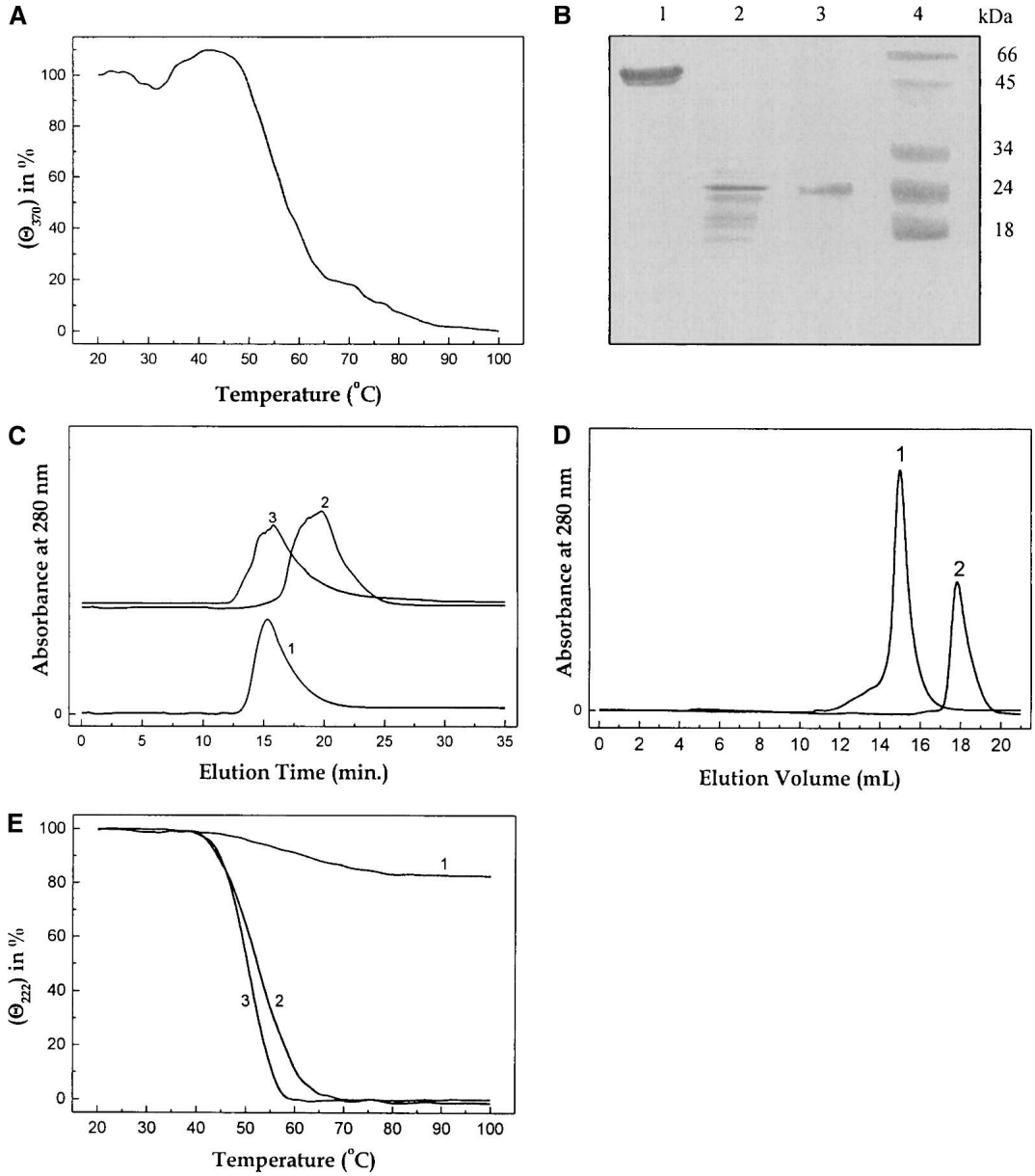

Figure 6. (*A*) Release of protein-bound FAD during thermal denaturation of FprA. Changes in the visible CD spectrum of FprA during thermal denaturation of the protein, as monitored by loss of the CD signal at 370 nm. The values are represented as percentage of the value observed for FprA sample at 20°C. (*B*) SDS-PAGE profiles of FprA (lane *1*), FprA after treatment with α-chymotrypsin (lane *2*), and purified NADP-binding domain of FprA (lane *3*). Lane *4* represents protein standards of molecular masses between 66 and 18 kDa. The conditions for proteolysis and purification of the NADP-binding domain are detailed in the Experimental Procedures section. (*C*) Affinity chromatography of FprA and the NADP-binding domain on HiTrap Blue Sepharose column. The curves 1 to 3 represent the elution profiles of FprA, and the FprA fragment in the absence and presence of 0.5 mM $NADP^+$, respectively. Experimental details are described in Experimental Procedures section. (*D*) Properties of the purified NADP-binding domain of FprA. Size-exclusion chromatographic profiles of FprA (curve 1) and of the purified NADP-binding domain of the enzyme (curve 2) on Superdex 200HR column at pH 7.0 and 25°C. (*E*) Thermal denaturation profiles of the NADP-binding domain of FprA at low ionic strength (curve 1); in the presence of 0.15 M NaCl (curve 2) and at pH 6 (curve 3) as measured by loss of the CD ellipticity at 222 nm. The values for loss of CD signal are represented as percentage of the value observed for protein sample at 20°C.

protein can be summarized as shown in Figure 7. Thermal denaturation of native FprA results in the unfolding of only the FAD-binding domain of the protein with release of the protein-bound FAD. The NADP-binding domain remains intact and folded under these conditions. The incubation of FprA with monovalent cation results in the compaction of

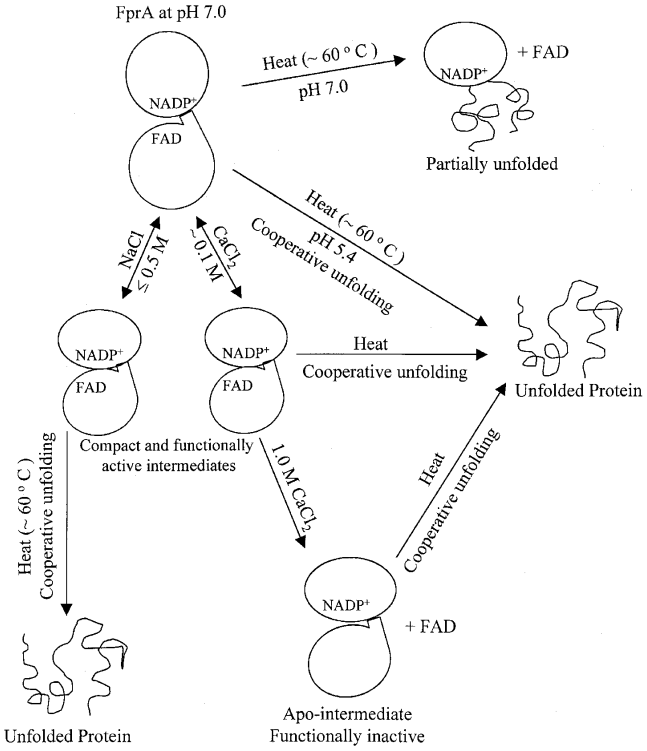

Figure 7. Diagrammatic scheme of the cation-induced structural and functional alteration in FprA.

the native conformation of the enzyme. This monovalent cation-stabilized compact conformation of FprA undergoes a complete cooperative unfolding on thermal denaturation. The divalent cation treatment of FprA stabilized two different intermediates, depending on the divalent cation concentration. The first intermediate, stabilized at very low divalent cation concentration, is a catalytically active, compact form of FprA, which might be similar to that found to be stabilized by monovalent cations (discussed above). However, at higher divalent cation concentrations, a catalytically inactive, apoprotein form, having molecular dimensions similar to that of the holoprotein, is stabilized. Both $CaCl_2$-stabilized intermediates of FprA showed a cooperative unfolding on thermal denaturation. As expected, the apoprotein form stabilized at higher $CaCl_2$ concentration showed a lower T_m on thermal denaturation than that observed for FprA in the absence of $CaCl_2$ or at low $CaCl_2$ concentration.

Electrostatic interactions among charged moieties of surface of ionizable residues contribute significantly to the conformational stability of proteins. The pH and cation dependence by stability is likely to be affected by the net charge on the protein, especially in highly charged proteins, in which long-range electrostatic interactions can contribute to the stability of native state. FprA is an acidic protein having an isoelectric point of about 5.5. For having an insight into the mechanism of cation- or pH-induced compaction of native conformation of FprA and subsequent induction of cooperativity in the protein molecule we studied the charge distribution on the protein surface. Figure 8 shows the electrostatic surface potential of FprA as obtained by GRASP (Nichollas 1992) from its crystal structure. The protein surface shows significant clustering of negative charges (Fig. 8), with a higher concentration of negative surface potential localized in the surface of FprA. Because of the large number of acidic residues, at pH 7.0, there are substantial repulsive long-range electrostatic interactions. The repulsion among the similar charges clustered in this region would be the main force that stabilizes the protein in a relatively open conformation. The interaction of cations with the negatively charged groups present in the protein will be helpful in screening, and hence weakening, of repulsive electrostatic forces present in a native conformation of the protein. Thus, a compaction of the protein will be favored in the presence of cations or acidic pH. The salt-

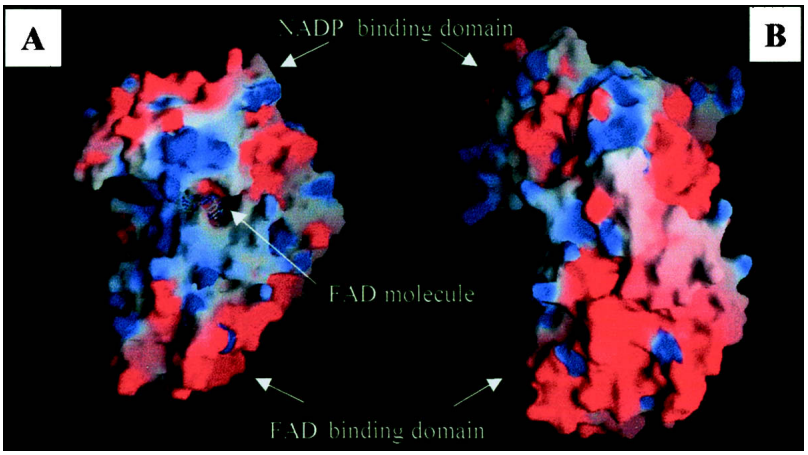

Figure 8. Molecular surface of FprA colored according to its electrostatic potential (blue for positive potential and red for negative potential). (*A*,*B*) The FprA molecule in different orientations. The surface was displayed using GRASP.

induced compaction of the protein may, in turn, lead to the observed enhanced cooperativity in its thermal unfolding.

A crucial problem in the designing of molecules, which can be effective drugs against a disease, is to achieve specificity for the protein/enzyme molecule of target organism. The importance of this problem lies in the fact that in most of the cases the conformational and physical properties of the host and the pathogen protein are not very different, and hence, it is a small difference, which can be exploited to bring about specificity for a molecule as a specific inhibitor of pathogen protein. In this regard correct information on the conformation and physical characteristics of the pathogen protein is of significant importance. Factors like pH and presence of ions are known to influence the protein conformation and physical properties, especially in the case of charged proteins. Due to this fact the role of the microenvironment in which the pathogen grows becomes important, especially in case of pathogen like *M. tuberculosis*, which grows in a hostile environment of the phagosomes. *M. tuberculosis* grows within the phagocytic vacuole of macrophages, which has a relatively hostile environment with limited nutrients and acidic pH. Although *M. tuberculosis* limits the acidification of phagosomes by recruiting a transport system which exchanges protons for cations like Na^+ and Mg^{2+}, the vacuole is still moderately acidic with a pH of about 6.1 (Xu et al. 1994). Studies on the influence of pH and divalent cations on the growth of *Mycobacteria* has demonstrated that growth of *M. tuberculosis* is extremely sensitive to acidic pH, and indicate that the organism acquires sufficient Mg^{2+} in order to grow in a mildly acidic environment such as that present in the phagocytic vacuole of the macrophages (Piddington et al. 2000). The results of the studies presented in this paper demonstrate that the conformation, stability, and cooperativity of the *M. tuberculosis* protein are significantly changed in the microenvironment in which the pathogen grows and proliferates. Such drastic changes in protein conformation and physical properties will have significant consequences on the inhibition properties of the molecule being used as specific inhibitor of the enzyme.

Materials and methods

Materials

All the chemicals were purchased from Sigma Chemical Co., and were of highest purity available.

Methods

Overexpression and purification of FprA

The overproduction in *Escherichia coli* and purification of recombinant FprA was carried out as described earlier (Fischer et al. 2002). The purified FprA showed >95% purity, as seen by ESI-MS and SDS-PAGE analysis.

Incubation of FprA with different salts

FprA (7 μM) was dissolved in 50 mM sodium phosphate (pH 7.0), in the absence and presence of increasing concentrations of NaCl, KCl, or CsCl, and incubated for 4 h at 4°C before the measurements were made. For $CaCl_2$ or $MgCl_2$ incubations conditions were as above, with the difference that sodium phosphate buffer was replaced by 20 mM Tris-HCl (pH 7.0).

pH denaturation of FprA

FprA (7 μM) was incubated in the presence of 10 mM phosphate buffer at various pH values in the range of 6.0–7.0 for 5 h at 4°C. The pH of the solution was maintained throughout the studies.

Enzyme assay

Diaphorase activity of the enzyme was measured at 25°C in sodium phosphate or Tris-HCl buffers using $K_3Fe(CN)_6$ as an electron acceptor and NADPH as a reductant as described earlier (Fischer et al. 2002). When investigating the effect of salts, the salt under study was included into the assay mixture, at the same concentration as present in the enzyme incubation solution.

Fluorescence spectroscopy

Fluorescence spectra were recorded with a Perkin-Elmer LS 50B spectrofluorometer in a 5 mm path-length quartz cell. The excitation wavelength for tryptophan and FAD fluorescence measurements were 290 nm and 370 nm, respectively. Emission spectra were recorded from 300 nm to 400 nm, and from 400 nm to 600 nm, respectively.

Circular dichroism

CD measurements were made with a Jasco J800 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results were expressed as the mean residual ellipticity [θ], which is defined as $[\theta] = 100 \times \theta_{obs}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in mol residue l^{-1}, and l is the length of the light path in centimeters. The CD spectra were recorded in a 1-mm cell at 25°C at an enzyme concentration of 7 μM and 100 μM for the far- and near-UV or visible regions, respectively. The values obtained were corrected by subtracting the baseline recorded in the absence of FprA under the same conditions. For thermal denaturation studies a scan rate of 60°C/min was used.

Size-exclusion chromatography

Gel filtration experiments were carried out on a Superdex 200HR 10/30 column (manufacturer's exclusion limit for proteins: 600 kDa) on an ÄKTA FPLC (Amersham Biosciences). The column was equilibrated and run in 50 mM sodium phosphate buffer (pH 7.0), containing the desired NaCl concentration, at 25°C at a flow rate of 0.3 mL/min. For studies using $CaCl_2$, the column was equilibrated and run at 25°C with 20 mM Tris-HCl (pH 7.0), containing the desired $CaCl_2$ concentration.

Limited proteolysis

FprA at a 0.5 mg/mL concentration was subjected to limited proteolysis by α-chymotrypsin at a protein to protease ratio of 50:1. Digestion mixture was incubated for 1 h at 45°C in 100 μL

reaction volume. The reaction was stopped by adding a protease inhibitor cocktail. The samples were analyzed by SDS-PAGE on a 15% polyacrylammide gel (Laemmli 1970).

Purification of the NADP-binding domain

The purification of the heat-stable domain of FprA was carried out by affinity chromatography using a HiTrap Blue Sepharose pre-packed 5 mL column (Amersham Biosciences). The column was equilibrated with 50 mM sodium phosphate (pH 7.4), using the ÄKTA-FPLC system (Amersham Biosciences) at 25°C. Native and α-chymotrypsin proteolysed FprA samples were separately loaded on to the column and eluted with a linear gradient from 0 to 1.5 M NaCl in the equilibration buffer at a flow rate of 2 mL/min. For chromatography in the presence of $NADP^+$, 0.5 mM $NADP^+$ was present in both the mobile phase and protein samples. The purity of the eluted protein samples was checked by SDS-PAGE (Laemmli 1970).

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