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メタデータ	言語: eng
	出版者:
	公開日: 2018-06-08
	キーワード (Ja):
	キーワード (En):
	作成者:
	メールアドレス:
	所属:
URL	https://doi.org/10.24517/00049539

C-terminal residues of ferredoxin-NAD(P)$^+$ reductase from *Chlorobaculum tepidum* are responsible for reaction dynamics in the hydride transfer and redox equilibria with NADP$^+$/NADPH

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Keywords

Flavoenzyme; stopped-flow; green sulfur bacteria; charge transfer.

Abbreviations

A, absorbance; AdR, adrenodoxin reductase; *Bs*, *Bacillus subtilis*; *Ct*, *Chlorobaculum tepidum*; CT, charge transfer; CTC, charge transfer complex; *Ec*, *Escherichia coli*; FAD, flavin adenine dinucleotide; Fd, ferredoxin; FMN, flavin mononucleotide; FNR, ferredoxin-NAD(P)$^+$ oxidoreductase; GR, glutathione reductase; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_d, dissociation constant; K_M, Michaelis constant; MALDI-TOF MS, matrix assisted laser dye ionization-time of flight mass spectrometry; PdR, putidaredoxin reductase; Tris, tris(hydroxymethyl)aminomethane; TrxR, bacterial NADPH-thioredoxin reductase; Suffixes: ox, oxidized; red, reduced; WT, wild type.

Abstract

Ferredoxin-NAD(P)$^+$ reductase ([EC 1.18.1.2], [EC 1.18.1.3]) from *Chlorobaculum tepidum* (*Ct*FNR) is structurally homologous to the bacterial NADPH-thioredoxin reductase (TrxR), but possesses a unique C-terminal extension relative to TrxR that interacts with the isoalloxazine ring moiety of the flavin adenine dinucleotide prosthetic group. In this study, we introduce truncations to the C-terminal residues to examine their role in the reactions of *Ct*FNR with NADP$^+$ and NADPH by spectroscopic and kinetic analyses. The truncation of the residues from Tyr326 to Glu360 (the whole C-terminal extension region), from Phe337 to Glu360 (omitting Phe337 on the *re*-face of the isoalloxazine ring) and from Ser338 to Glu360 (leaving Phe337 intact) resulted in a blue-shift of the flavin absorption bands. The truncations caused a slight increase in the dissociation constant toward NADP$^+$ and a slight decrease in the Michaelis constant toward NADPH in steady-state assays. Pre-steady-state studies of the redox reaction with NADPH demonstrated that deletions of Tyr326–Glu360 decreased the hydride transfer rate, and the amount of reduced enzyme increased at equilibrium relative to wild-type *Ct*FNR. In contrast, the deletions of Phe337–Glu360 and Ser338–Glu360 resulted in only slight changes in the reaction kinetics and redox equilibrium. These results suggest that the C-terminal region of *Ct*FNR is responsible for the formation and stability of charge-transfer complexes, leading to changes in redox properties and reactivity toward NADP$^+$/NADPH.

Introduction

Ferredoxin-NAD(P)$^+$ oxidoreductase ([EC 1.18.1.2], [EC 1.18.1.3], FNR), adrenodoxin reductase ([EC 1.18.1.6], AdR) and putidaredoxin reductase ([EC 1.18.1.5], PdR) are NAD(P)H-dependent oxidoreductases in the flavoprotein superfamily that catalyze the redox reaction between the two-electron carrier nucleotides NAD(P)$^+$/NAD(P)H, and the one-electron carrier iron-sulfur proteins ferredoxin (Fd), adrenodoxin, and putidaredoxin, respectively. These enzymes have a conserved protein topology composed of two nucleotide-binding domains: one for flavin adenine dinucleotide (FAD)- or flavin mononucleotide (FMN)-binding, and the other for NAD(P)$^+$/NAD(P)H-binding (Aliverti et al. 2008; Dym and Eisenberg 2001). Based on amino acid sequence and structure analyses, FNR, AdR and PdR can be classified into two major categories, plant-type and glutathione reductase (GR)-type, each of which includes several subgroups (Aliverti et al. 2008; Correll et al. 1993; Karplus and Faber 2004; Ceccarelli et al. 2004; Medina and Gomez-Moreno 2004; Seo et al. 2004). The TrxR-type FNR, a subgroup of the GR-type FNR found in green sulfur bacteria, Firmicutes and several Alphaproteobacteria, is distinctive from the other types of FNR as it is a homodimer with significant conservation of structural topology with bacterial NADPH-thioredoxin reductase (TrxR) (Muraki et al. 2010; Komori et al. 2010). The crystal structures of *Chlorobaculum tepidum* FNR (*Ct*FNR, Muraki et al. 2010 [PDB code: 3AB1]), *Thermus thermophilus* HB8 thioredoxin reductase-like protein (PDB code: 2ZBW) and *Bacillus subtilis* FNR (*Bs*FNR, Komori et al. 2010 [PDB codes: 3LZX, 3LZW]) revealed stacking of aromatic residues on the *si*- and *re*-faces of the isoalloxazine ring moiety and a C-terminal extension relative to TrxR on the *re*-face of the ring. In the crystal structure of oxidized *Ct*FNR, Tyr57 and Phe337 stacked on the *si*- and *re*-faces, respectively, of the isoalloxazine ring moiety, almost in parallel, at a distance of approximately 3.5 Å from the centroid of the isoalloxazine ring. The 23 amino acid residues following Phe337 comprise the 7th α-helix of the protein, form the C-terminal extension, and are on the *re*-face of the ring (Fig. 1a, b, Muraki et al. 2010). Interestingly, similar arrangements of amino acid residue(s) around the isoalloxazine ring moiety are often found in the flavoprotein superfamily, including plant-type FNRs. In plant-type FNRs, mutations of the amino acid residues close to the *re*-face of the isoalloxazine ring moiety, including the *re*-face Tyr residue, resulted in drastic changes in the specificity, reactivity and the relative redox properties toward NAD(P)$^+$/NAD(P)H (Piubelli et al. 2000; Nogués et al. 2004; Tejero et al. 2005; Lans et al. 2010; Peregrina et al. 2010; Bortolotti et al. 2014). Steady-state studies with truncations of the C-terminal extension region of *Bs*FNR, a TrxR-type FNR, revealed that the truncations had little effect on the NADPH oxidation reaction, but the rate of Fd reduction decreased drastically (Seo et al. 2014). However, the details concerning the role of the C-terminal region of the TrxR-type FNR, especially in the hydride transfer process and formation of the NAD(P)$^+$/NADPH-FNR complex, remain unclear.

Recently, pre-steady-state kinetic studies of *Bs*FNR and *Ct*FNR have revealed that hydride transfer is the rate-limiting step during the NADPH oxidation reaction, and the rate constant of the hydride transfer step toward NADPH oxidation is much faster than that toward NADP$^+$ reduction (500 s^{-1} vs. <10 s^{-1}) in the case of *Bs*FNR, whereas the rate constants in both directions are comparable in the case of *Ct*FNR (Seo et al. 2016a, b). As this difference in reversibility relates to their physiological roles, a comparative study of these two FNRs would contribute to our understanding of the structure-function relationships of FNRs. In the structures of these two FNRs, there are conspicuous differences at the C-terminal extension (Fig. 1a, b), in the NADPH binding site on the surface of the NAD(P)$^+$/NAD(P)H-binding domain, and in the hinge regions that connect the two nucleotide-binding domains (Muraki et al. 2010; Komori et al. 2010). The crystal structure of *Bs*FNR in the NADP$^+$-bound form revealed that *Bs*FNR and *Escherichia coli* (*Ec*) TrxR share similarities in their NADP$^+$ binding mode (Komori et al. 2010), leading to the idea that a drastic domain motion must occur to achieve a hydride transfer between NAD(P)$^+$/NAD(P)H and the FAD prosthetic group (Waksman et al. 1994). Therefore, the above differences between *Bs*FNR and *Ct*FNR would affect the reactivity toward NADP$^+$/NADPH to some extent. The C-terminal extension region would affect the close stacking of the nicotinamide ring moiety of NADP$^+$/NADPH with the isoalloxazine ring moiety of FAD, thereby participating in the formation of a charge transfer complex (CTC) with NAD(P)$^+$/NAD(P)H. From the alignment of the amino acid sequences of the C-terminal regions of TrxR-type FNRs, *Ct*FNR has a long, hydrophilic residue-rich C-terminal region, while that of *Bs*FNR is shorter (Fig. 1c). The differences in the C-terminal region may relate to the differences in catalysis between *Bs*FNR and *Ct*FNR with NADP$^+$/NADPH.

To analyze the role of the C-terminal extension in *Ct*FNR during the redox reaction with NAD(P)$^+$/NAD(P)H, we performed steady-state and pre-steady-state kinetic analyses of *Ct*FNR mutants with specific alterations designed to truncate the C-terminal region. The results obtained suggest that the C-terminal region controls the positioning of the nicotinamide ring portion of NADP$^+$/H against the isoalloxazine ring portion of the FAD prosthetic group, leading to changes in the redox properties and reactivity toward NADP$^+$/NADPH.

Materials and methods

Preparation of WT and mutant *Ct*FNRs

Expression vectors for the truncation mutants of the C-terminal residues were prepared by replacement of the codons for Tyr326, Phe337 or Ser338 with a stop codon (TAA) by QuikChange site directed mutagenesis, utilizing the wild-type (WT) *Ct*FNR expression vector (Muraki et al. 2008) as the template and with the following primers; 5′-CCACAGCCTCTCCTAAATCAAGCCGGGCGAG-3′ and 5′-CTCGCCCGGCTTGATTTAGGAGAGGCTGTGG-3′ for *Δ*Tyr326–Glu360, 5′-GAAAATTCGTAATGTCTAAAGCAGCGTCAAGATGGCCAAG-3′ and 5′-CTTGGCCATCTTGACGCTGCTTTAGACATTACGAATTTTC-3′ for *Δ*Phe337–Glu360 or 5′-GAAAATTCGTAATGTCTTCTAAAGCGTCAAGATGGCCAAG-3′ and 5′-CTTGGCCATCTTGACGCTTTAGAAGACATTACGAATTTTC-3′ for *Δ*Ser338–Glu360. The DNA sequence of the open reading frame of the mutated *Ct*FNR gene was verified at the Institute for Genomic Research, Kanazawa University. The plasmids obtained were transformed into *E. coli* Tuner (DE3) pLacI cells (Novagen, Merck-Millipore, Merck KGaA, Germany). Expression and purification of mutated *Ct*FNRs were performed using the same methods as for WT *Ct*FNR (Seo et al. 2016b) except utilizing a Source 30Q anion exchange gel column (GE Healthcare UK Ltd., England) in place of a MonoQ column during the purification of *Δ*Phe337–Glu360 *Ct*FNR. WT *Ct*FNR was prepared as described in Seo et al. (2016b).

Steady-state enzyme assays

The NADPH diaphorase activity using potassium ferricyanide as the electron acceptor was measured in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH buffer (pH 7.0) in the presence of 10 mM glucose-6-phosphate (G6P; Oriental Yeast Co., Ltd., Tokyo, Japan), 5 U/ml glucose-6-phosphate dehydrogenase (G6PDH, *Leuconostoc mesenteroides*; Biozyme Laboratories, Blaenavon, UK), 10 nM FNR and 2 mM potassium ferricyanide, together with 0–1 mM NADPH (Oriental Yeast Co., Ltd.) at 25°C under aerobic conditions, by monitoring the decrease in absorbance at 420 nm for 1 min using a double beam spectrophotometer (V-560, JASCO Co., Tokyo, Japan). The respective assay blank containing all of the assay reagents except FNR was subtracted for turnover rate estimation. Turnover rates are expressed as the number of NADPH molecules consumed by one homo-dimeric FNR. The Michaelis constant (K_M) and k_{cat} values were evaluated by nonlinear regression analysis using the Michaelis-Menten equation in Igor Pro software (ver. 6.3, WaveMetrics, Portland, OR, USA).

Stopped-flow spectrophotometry

Stopped-flow spectrophotometry was performed using a stopped-flow system with a photodiode array detector (Unisoku Co., Ltd., Osaka, Japan) in a glove box under a nitrogen atmosphere containing approximately 5% hydrogen as described in Seo et al. (2016a). The reaction was initiated by mixing equal volumes of solutions in single-mixing mode at 10°C. Transient spectra were recorded every 1 ms after mixing. The dead-time of the setup was estimated to be approximately 1 ms (Seo et al. 2016a). Concentrations of the *Ct*FNR protomers and NADP$^+$/NADPH/(4S-2H)-NADPD are provided as the final concentrations after mixing, unless otherwise noted.

Reduced *Ct*FNR (*Ct*FNR$_{red}$) was prepared with dithionite in the presence of methyl viologen followed by the removal of the latter by size-exclusion chromatography, as described previously (Seo et al. 2016b). The concentration of reduced *Ct*FNR was estimated from the absorbance of the air-reoxidized form.

Data collection and basic arithmetic operations on the transient spectra were performed using Unispec (ver. 2.51, Unisoku Co., Ltd.) and Excel (ver. 15, Microsoft Corporation, Redmond, WA, USA) software. The transient absorptions at a single wavelength were fitted to exponential decay functions in Igor Pro software. The values of the absorbance at 457 nm (A_{457}) and A_{590} in Figs. 3b, 3d, 3f, 4b, 4d and 5b were estimated by subtracting A_{800} to compensate for the signal drift. Global analysis of the transient absorption data was performed with Olis GlobalWorks software (ver. 5.888.179, Olis Inc, USA). The selection of potential kinetic reaction models was made based on the weight values, and the spectral and kinetic eigenvectors of the components estimated by a singular value decomposition analysis. Model validity was assessed by kinetic and overall standard deviations of the fitting.

Miscellaneous methods

UV-visible absorption spectra were measured with a double beam spectrophotometer (V-560, JASCO) at 23 or 25°C. NADP$^+$ titration was performed by the previously described method in 20 mM HEPES-NaOH buffer (pH 7.0) at 23 or 25°C (Seo et al. 2014). Difference spectra were obtained by subtracting the control spectrum recorded prior to the addition of NADP$^+$ from the experimentally obtained spectra after correcting for volume changes. Dissociation constant (K_d) values were calculated according to Batie and Kamin (1984).

Thermal stabilities of the non-covalently bound FAD prosthetic group in the WT and mutated *Ct*FNRs in oxidized form were measured by the previously described methods (Seo et al. 2015). *Ct*FNR solution (~10 μM) in 20 mM HEPES-NaOH buffer (pH 7.0) was incubated at each temperature

over a range from 20 to 90°C for 5 min and then cooled on ice. After 20 min of centrifugation at 16,000 $\times g$, the supernatant was retained. Fluorescence intensity of the supernatant with excitation at 450 nm and emission at 525 nm was measured on a fluorescence spectrophotometer (F-2500, Hitachi, Japan). Data were fitted to a simple two-state transition model as described by John and Weeks (2000).

SDS-PAGE analysis was performed as described by Laemmli (1970). Protein bands on a 12% acrylamide gel were visualized with Coomassie Brilliant Blue R-250. Molecular weight marker (Precision Plus Protein Standard) was purchased from BioRad Lab. Inc., Japan.

Molecular masses of native forms of the enzymes were deduced by gel-permeation chromatography on a Superdex-200 10/300 column (GE Healthcare UK Ltd.) at a flow rate of 0.4 ml min^{-1} utilizing 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.0) containing 200 mM NaCl as the eluent and molecular mass standards purchased from Sigma-Aldrich (Kit for Molecular Weights 12,000–200,000, MWGF200; St Louis, MO, USA).

Matrix assisted laser dye ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis was performed utilizing an Ab Sciex 4800 Plus MALDI TOF/TOF^{TM} analyzer (Sciex, Framingham, MA, USA) in the linear mode with sinapic acid as the matrix. Each data point was expressed as the mean value of the 40 times of integration of 50 shots at a laser intensity of 3000. Bovine serum albumin was used as a molecular mass standard.

Absorption coefficients for mutated CtFNRs were determined by the heat denaturation method as previously described (Aliverti et al. 1999; Seo et al. 2014). The extinction coefficient of 11.3 mM^{-1} cm^{-1} at 450 nm was utilized for estimation of FAD concentration.

(4S-2H)-NADPD (the deuterated form of NADPH, referred to hereafter as S-NADPD) was prepared according to the method described in Seo et al. (2016a).

Protein and substrate concentrations were determined using the following extinction coefficients: WT CtFNR ($\varepsilon_{466} = 10.3$ mM^{-1} cm^{-1}, Seo and Sakurai 2002), ΔTyr326–Glu360 CtFNR ($\varepsilon_{457} = 10.3$ mM^{-1} cm^{-1}), ΔPhe337–Glu360 CtFNR ($\varepsilon_{457} = 10.6$ mM^{-1} cm^{-1}), ΔSer338–Glu360 CtFNR ($\varepsilon_{458} = 10.3$ mM^{-1} cm^{-1}), potassium ferricyanide ($\varepsilon_{420} = 1.02$ mM^{-1} cm^{-1}), and NADPH ($\varepsilon_{340} = 6.2$ mM^{-1} cm^{-1}). NADP$^+$ concentration was determined from the reduced form in the presence of excess G6P and G6PDH.

Results

Preparation and spectroscopic and molecular properties of WT and mutated *Ct*FNRs

Three C-terminally truncated *Ct*FNRs, *Δ*Tyr326–Glu360 *Ct*FNR, *Δ*Phe337–Glu360 *Ct*FNR and *Δ*Ser338–Glu360 *Ct*FNR (hereafter designated *Δ*Y326 *Ct*FNR, *Δ*F337 *Ct*FNR and *Δ*S338 *Ct*FNR, respectively) were purified to homogeneity (Fig. S1). The WT *Ct*FNR polypeptide migrated with an apparent molecular mass of 42 kDa on SDS-PAGE (Fig. S1, Seo and Sakurai 2002). The apparent molecular masses of *Δ*Y326, *Δ*F337 and *Δ*S338 *Ct*FNRs were approximately 38, 39 and 39 kDa based on SDS-PAGE, respectively (Fig. S1). MALDI-TOF MS analysis of WT *Ct*FNR in the native form produced a major peak with a mass of 39.19 kDa (calculated mass, 39240 Da by sequence without FAD) (Fig. S2). *Δ*S338 showed a peak at 36.93 kDa (calculated mass, 36864 Da by sequence without FAD), and *Δ*Y326 at 35.33 kDa (calculated: 35420 Da by sequence without FAD) (Fig. S2). All the purified *Ct*FNRs eluted as a single peak during gel-permeation chromatography. The deduced apparent molecular mass of WT *Ct*FNR was approximately 91 kDa (Table 1), and the apparent molecular masses of *Δ*Y326, *Δ*F337 and *Δ*S338 *Ct*FNRs were 68 kDa, 66kDa and 71 kDa, respectively (Table 1).

The UV-visible absorption spectra of *Δ*Y326, *Δ*F337, and *Δ*S338 *Ct*FNRs exhibited FAD transition bands I and II in the near-UV to visible region with peaks centered around 456–458 nm and 378–381 nm, respectively (Fig. 2a). The absorption coefficients of WT and mutated *Ct*FNRs at the λ_{max} of the FAD transition band I (456–466 nm) exhibited similar values (10.3-10.6 mM^{-1} cm^{-1}) (Fig. 2a, Table 1).

The thermal stabilities of bound FAD in the WT and the truncated *Ct*FNRs were examined by measuring the flavin fluorescence emission of the retained supernatant after incubation and centrifugation (Fig. 2b). The temperatures providing 50% of the maximal difference in fluorescence intensity of *Δ*F337 and *Δ*S338 *Ct*FNRs were estimated to be 69°C and 70°C, respectively, which were similar to that of WT (70°C). On the other hand, that of *Δ*Y326 *Ct*FNR was estimated to be 62°C, which was 8°C lower than those of the WT *Ct*FNR.

Steady-state assays with NADP$^+$/NADPH

Addition of NADP$^+$ to a solution of WT *Ct*FNR induced blue-shifts and decreases in the flavin absorption bands, which produced troughs at approximately 514, 477, 445 and 404 nm in the difference spectrum (Fig. 2c). However, the addition of NADP$^+$ to *Δ*Y326, *Δ*F337 and *Δ*S338 *Ct*FNR solutions resulted in red-shifts and decreases in the bands, producing a peak at 504–505 nm and troughs at 477-478 and 450 nm in the difference spectra (Fig. 2c). Plots of the magnitude of spectral

changes, $\Delta A_{503-506}$ *minus* ΔA_{477} for ΔY326, ΔF337 and ΔS338 *Ct*FNRs, and ΔA_{495} *minus* ΔA_{514} for WT *Ct*FNR against NADP$^+$ concentration yielded saturation curves (Fig. 2d). The K_d values for NADP$^+$ for ΔY326, ΔF337 and ΔS338 *Ct*FNRs were slightly higher than that for WT *Ct*FNR (Table 1).

To evaluate the reactivity of the WT and truncated *Ct*FNRs with NADPH, the turnover rate of diaphorase activity was measured using potassium ferricyanide as the electron acceptor (Table 1). On truncation of the C-terminal extension, the k_{cat} and K_M values for NADPH for the three mutants decreased slightly when compared to those for WT *Ct*FNR (Table 1). As a result, the k_{cat}/K_M values of the truncation mutants were of the same order of magnitude as that of the WT *Ct*FNR.

Pre-steady-state reaction of ΔY326 *Ct*FNR$_{ox}$ with NADPH/*S*-NADPD

The reaction of 9.3 μM ΔY326 *Ct*FNR$_{ox}$ with 100–500 μM NADPH was measured by stopped-flow spectrophotometry (Fig. 3a, b). Mixing ΔY326 *Ct*FNR$_{ox}$ with NADPH resulted in a rapid decrease in the flavin absorption band I (peak centered around 457 nm) within the dead-time of the instrument (~1 ms). The absorbance of this band decreased to an almost fully-reduced level by 1999 ms (Fig. 3a). The absorbance change at 457 nm contains four distinctive kinetic components designated as phase I, II, III and IV in order of the rate constants from fastest to slowest (Fig. 3b).

Phase I, observed as a rapid drop within the dead-time, was too rapid to trace in our stopped-flow system (>500 s^{-1}). The Δabsorbance of phase I was almost the same at the NADPH concentrations used (100–500 μM; traces b–d in Fig. 3b). Mixing with *S*-NADPD in place of NADPH also resulted in a rapid drop with a similar Δabsorbance as that for NADPH (trace e in Fig. 3b).

After the rapid drop, A_{457} decreased gradually in the 1 to 1000 ms time period (Fig. 3b). The absorption change at 457 nm could be approximated by a three-step reaction model (red lines in Fig. 3b, Table 2). The observed rate constant (k_{obs}) of phase II (k_1 in Table 2), which appeared as a major decay component at 0–10 ms, became almost constant at NADPH concentrations above 200 μM with a value of ~200 s^{-1}. The amplitude of phase III (ΔA_2 in Table 2) increased with increasing NADPH concentration, and its k_{obs} (k_2 in Table 2) was nearly independent of the NADPH concentration used, with a value of ~30 s^{-1}. The k_{obs} of phase IV (k_3 in Table 2), observed as a slope after 200 ms, was estimated to be 3–3.5 s^{-1} using the data in the time range 0 to 999 ms.

Upon mixing with 100 μM *S*-NADPD in place of NADPH, the absorption change at 457 nm after the rapid drop could be approximated by a two-step reaction model (trace e in Fig. 3b). The rate of phase II was estimated to be 16.2 ± 0.4 s^{-1}, and that of the slower phase was uncertain because the absorption change continued beyond the measurement time scale.

In the reaction with NADPH and *S*-NADPD, A_{590} in the charge transfer (CT) band region (Seo et al 2016a, b) rapidly increased within the instrument dead-time and reached its maximum intensity by 2

ms (Fig. 3a, inset of Fig.3b). Subsequently, the absorption intensity of this CT band decreased over 999 ms without a significant peak shift (Fig. 3a). Use of higher NADPH concentration (100–500 μM) did not significantly affect the maximum absorption intensity of the CT band (inset of Fig. 3b). The A_{590} can be approximated by a single-step reaction model (inset of Fig.3b). Obtained k_{obs} values were 27.4 ± 0.8 s^{-1} at 100 μM NADPH and 26.9 ± 0.6 s^{-1} at 500 μM NADPH.

To clarify any kinetic components induced by the use of excess NADPH, mixing with equimolar NADPH (25 μM) was performed as a reference (Fig. 3c, d). The observed absorption change at 457 nm after the rapid drop could be approximated by a two-step reaction model (red line in Fig. 3d, Table 2). The faster phase had a comparable rate constant (117 s^{-1}) to that of phase Ⅱ with excess NADPH (100–500 μM), and the slower phase had a similar rate constant (3.1 s^{-1}) to that of phase Ⅳ in the excess NADPH experiments (Table 2)

Global analysis of the time-resolved absorption spectra implied that the reactions mixing with excess NADPH were approximated by a three-step sequential reaction model (A→B→C→D [fast/moderate/slow]) (inset of Fig.3a), and those with equimolar NADPH were approximated by a two-step sequential reaction model (A→B→C [fast/slow]) (inset of Fig. 3c). The resulting rate constant for each reaction step (represented in the parentheses in Table 2) was comparable to that of the calculated value from the single wavelength analysis. The resulting spectrum of each of the kinetic components is shown in the insets of Fig. 3a, 3c.

Pre-steady-state reaction of ∆Y326 *Ct*FNR$_{red}$ with NADP$^+$

Mixing *∆*Y326 *Ct*FNR$_{red}$ (10.6 μM) with NADP$^+$ resulted in a slow increase in the flavin absorption band I (Fig. 3e, f). The total absorption changes at near-equilibrium (i.e., at 1999 ms) exhibited a significant dependence on the NADP$^+$ concentration in the range of 100 to 500 μM (Fig. 3f). A_{457} in the 0 to 1999 ms time period could be approximated by a single-step reaction model (Fig. 3f). The k_{obs} values were estimated to be 6.05 ± 0.01, 5.45 ± 0.01 and 4.61 ± 0.01 s^{-1} at 100, 200 and 500 μM NADP$^+$, respectively. From $∆A_{457}$ at 3999 ms, the oxidation level of *Ct*FNR was estimated to be approximately 40% at 100 μM NADP$^+$ (Fig. 3e).

In contrast to the reaction of *∆*Y326 *Ct*FNR$_{ox}$ with NADPH (Fig. 3a), no significant absorption change was observed in the CT band region (550–800 nm) during the reaction in the NADP$^+$ concentration range of 100–500 μM (Fig. 3e).

The global analysis confirmed that the reactions mixing with NADP$^+$ were approximated by a single-step reaction model (A→B) with a rate constant of 6.04 ± 0.06 s^{-1} at 100 μM NADP$^+$ (inset of Fig.3e).

Pre-steady-state reactions of ∆S338 *Ct*FNR$_{ox}$ with NADPH/*S*-NADPD

The reaction of 9.3 μM ΔS338 CtFNR$_{ox}$ with NADPH resulted in a rapid decrease in the flavin absorption band I (peak centered around 457 nm) within the dead-time of the instrument (~1 ms), subsequently, the absorbance of this band decreased gradually to around 40% of that of ΔS338 CtFNR$_{ox}$ at 3999 ms (Fig. 4a). The analyses of the absorbance change at 457 nm with NADPH and S-NADPD contained four distinctive kinetic components, designated as phase I, II, III and IV in order of the rate constants from fastest to slowest (Fig. 4b).

The Δabsorbance of the rapid absorption drop within the dead-time at 457 nm was similar at the NADPH concentration used (100–500 μM) (trace b-d in Fig. 4b). Although mixing with 100 μM S-NADPD in place of NADPH also resulted in a rapid drop within the dead time (trace e in Fig. 4b), its Δabsorbance was smaller than that with NADPH (trace b in the inset of Fig. 4b). After the drop, an additional small decay component appeared in the 0 to 5 ms time period (trace e in the inset of Fig. 4b). Corresponding to the results of mixing ΔY326 CtFNR$_{ox}$ with NADPH, the former S-NADPD-independent rapid drop is designated as phase I and the latter S-NADPD dependent decay is designated as phase II in the Discussion section.

After the rapid drop with NADPH, A_{457} decreased gradually in the 1 to 1000 ms time period (Fig. 4b). The absorption change with NADPH in this time period could be approximated by a two-step reaction model (red lines in Fig. 4b). The Δabsorbance of phase III, a major decay component in the 1 to 100 ms time period, increased with increasing NADPH concentration, and k_{obs} of this phase decreased with increasing NADPH concentration (ΔA_1 and k_1 in Table 3). The absorption change at 457 nm upon mixing with 100 μM S-NADPD could be approximated by a three-step reaction model (trace e in Fig. 4b); k_{obs} of phase II was estimated to be 310 ± 12 s^{-1}, and phase III was estimated to be 33.7 ± 0.7 s^{-1}. The k_{obs} of phase IV (k_2 in Table 3), observed as a slope beyond 100 ms with both NADPH and S-NADPD, was uncertain, as this phase continued beyond the measurement time scale.

In the reaction of ΔS338 CtFNR$_{ox}$ with NADPH and S-NADPD, the absorbance around 600 nm which corresponds to the formation of CTCs (Seo et al 2016a, b), rapidly increased within the dead-time and reached its maximal intensity by 5 ms (Fig. 4a). Subsequently, the intensity of this absorption band decreased slightly with time, without a significant peak shift by 3999 ms (Fig. 4a). The NADPH concentration used (100–500 μM) hardly affected the maximum absorption intensity of the CT band.

Global analysis of the transient absorption indicated that the reaction mixing with excess NADPH was approximated by a three-step sequential reaction model (A→B→C→D [fast/moderate/slow]) with similar rate constants to the single wavelength analysis except for the presence of an additional faster phase (represented in the parentheses in Table 3). The resulting spectrum of each kinetic component is shown in the inset of Fig. 4a.

Pre-steady-state reaction of ΔS338 *Ct*FNR$_{red}$ with NADP$^+$

Mixing *ΔS*338 *Ct*FNR$_{red}$ (11.7 μM) with NADP$^+$ resulted in a slow increase in the flavin absorption band I (Fig. 4c, d). The total absorption change at near-equilibrium (i.e., at 1999 ms) showed a significant dependence on the NADP$^+$ concentration in the range of 100–500 μM (Fig. 4d). In the 0 to 1999 ms time period, A_{457} could be approximated by a two-step reaction model (red lines in Fig. 4d). The k_{obs} values of the faster phase were estimated to be 7.70 ± 0.02, 7.04 ± 0.018 and 5.94 ± 0.02 at 100, 200 and 500 μM NADP$^+$, respectively. As the absorption change continued beyond the measurement timescale, the k_{obs} value of the slower phase was uncertain (<4 s^{-1}). From ΔA_{457} at 3999 ms, the oxidation level of *ΔS*338 *Ct*FNR was estimated to be approximately 60% at 100 μM NADP$^+$ (Fig. 4c). No significant absorption change was observed in the CT band region (550–800 nm) during the reaction, in the 100–500 μM NADP$^+$ concentration range (Fig. 4c). Global analysis implied that the reaction mixing with NADP$^+$ was approximated by a two-step sequential reaction model (A→B→C [fast/slow]) with rate constants of 7.70 ± 0.19 and 1.14 ± 0.09 for the first and second reaction steps, respectively, at 100 μM NADP$^+$. The spectrum of each of the kinetic components is shown in the inset of Fig.4c.

Pre-steady-state reactions of ΔF337 *Ct*FNR$_{ox}$ with NADPH/*S*-NADPD

To determine if the difference in the pre-steady state kinetics of the NADPH/*S*-NADPD oxidation reaction between ΔY326 *Ct*FNR and ΔS338 *Ct*FNR corresponds to the absence of Phe337, we prepared ΔF337 *Ct*FNR and evaluated the effect of this mutation on the NADPH/*S*-NADPD oxidation reactions by stopped-flow spectrophotometry (Fig. 5a, b).

The reaction of 9.0 μM *Δ*F337 *Ct*FNR$_{ox}$ with NADPH resulted in a rapid decrease in the flavin absorption band I within the dead-time and then the absorbance of this band decreased gradually (Fig. 5a). The absorption changes at 457 nm upon mixing with NADPH and *S*-NADPD contained four distinctive kinetic components, designated as phase I, II, III and IV in order of the rate constants from fastest to slowest (Fig. 5b).

Although the *Δ*absorbance of the rapid absorption drop at 457 nm was similar at all of the NADPH concentrations used (100–500 μM) (trace b-d in Fig. 5b), the *Δ*absorbance of mixing with 100 μM *S*-NADPD (trace e in the inset of Fig. 5b) was smaller than that of mixing with 100 μM NADPH (trace b in the inset of Fig. 5b). After the drop, an additional small decay component appeared in the 0 to 5 ms time period (trace e in the inset of Fig. 5b) just as with *ΔS*338 *Ct*FNR (Fig. 4b). After the rapid drop, A_{457} decreased gradually in the 2 to 1000 ms time period for NADPH (or in the 5 to 1000 ms time period for *S*-NADPD) (Fig. 5b). In the reaction with NADPH, the absorption change in this time period could be approximated by a two-step reaction model (red lines in Fig. 5b). With increasing

NADPH concentration, the Δabsorbance of phase Ⅲ, a major decay component observed in the 2 to 100 ms time period increased, and k_{obs} of this phase decreased (ΔA_1 and k_1 in Table 4). The absorption change upon mixing with 100 μM S-NADPD could be approximated by a three-step reaction model (trace e in Fig. 5b). The k_{obs} values of phases Ⅱ and Ⅲ were estimated to be 239 ± 6 s^{-1} and 14.7 ± 0.2 s^{-1}, respectively (trace e in Fig. 5b). k_{obs} of phase Ⅳ (k_2 in Table 4), observed as a slope beyond 100 ms with both NADPH and S-NADPD, was uncertain, as this phase continued beyond the measurement time scale.

In the reaction of ΔF337 CtFNR$_{ox}$ with NADPH and S-NADPD, the absorbance around 600 nm which corresponds to the formation of CTCs (Seo et al 2016a, b) rapidly increased within the dead-time and reached its maximum intensity by 5 ms (Fig. 5a). Subsequently, the intensity of this absorption band decreased slightly with time, without a significant peak shift by 3999 ms (Fig. 5a).

Global analysis of the transient spectra indicated that the reactions with NADPH were approximated by a two-step sequential reaction model (A→B→C (fast/slow)) with similar rate constants to those of the single wavelength analyses (Table 4). The resulting spectrum for each kinetic component is shown in the inset of Fig. 5a.

Discussion

In this study, ΔY326, ΔF337 and ΔS338 C-terminal truncation variants of *Ct*FNR showed apparent molecular masses corresponding to a homodimeric form, as was also observed for WT *Ct*FNR (Table 1). Compared with the flavin absorption bands of the WT *Ct*FNR, those of the truncation mutants shifted toward shorter wavelength (Fig. 2a), indicating that the truncations perturbed the environment of the FAD isoalloxazine ring. The truncation of the corresponding region in *Bs*FNR and replacement of Phe337 in *Ct*FNR also resulted in blue-shifts of the flavin absorption bands (Muraki et al. 2010; Seo et al. 2014). The difference spectra of the truncation mutants induced by the addition of NADP$^+$ were similar to that of the Phe337Ser mutant rather than those of Phe337His and Phe337Tyr mutants (Muraki et al. 2010). These indicate a loss of hydrogen bonding, a change in the polarity and/or a loss of the dipole field of the C-terminal helix could cause the observed spectral changes (Yagi et al. 1980; Heelis 1982). Although the truncations induce substantial changes in the environment of the FAD isoalloxazine ring, the thermal stability of the bound FAD prosthetic group was not drastically modulated (Fig. 2b). In steady-state reactions with NADP$^+$ and NADPH, K_d, K_M and k_{cat} values of the truncated *Ct*FNRs were similar (70–180% of those of WT *Ct*FNR) (Table 1). Pre-steady-state kinetics, however, indicated that the truncation mutations significantly altered the kinetics and equilibria of the reactions with NADP$^+$/NADPH.

In the NADPH oxidation reaction with ΔS338 and ΔF337 *Ct*FNRs$_{ox}$, the CT absorption band centered around 600 nm appeared within the experimental dead-time and reached its maximal intensity by 5 ms. The NADPH concentration used did not significantly affect the maximal intensity. These results are consistent with the rapid formation of Michaelis complex 1 (FNR$_{ox}$_NADPH, MC-1) before the formation of CTC-1 (Scheme 1; Lennon and Williams Jr. 1997, Tejero et al. 2007; Seo et al. 2016a, b).

$$\underset{\text{MC-1}}{} \quad \underset{\text{CTC-1}}{} \quad \underset{\text{CTC-2}}{} \quad \underset{\text{MC-2}}{}$$

$$\text{FNR}_{ox} + \text{NADPH} \rightleftarrows \text{FNR}_{ox}\_\text{NADPH} \rightleftarrows \text{FNR}_{ox}\text{-NADPH} \underset{k_f}{\overset{k_r}{\rightleftarrows}} \text{FNR}_{red}\text{-NADP}^+ \rightleftarrows \text{FNR}_{red}\_\text{NADP}^+ \rightleftarrows \text{FNR}_{red} + \text{NADP}^+$$
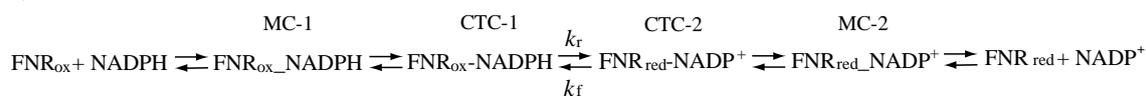
Scheme 1

When *S*-NADPD was used in place of NADPH, a small decay component was observed from 0 to 5 ms (phase II) (trace e in Fig. 4b, 5b), indicating that phase II was affected by the deuterium substitution. Because k_{obs} decreased and the Δabsorbance increased with increasing NADPH concentration, phase III corresponds to the formation of the FNR$_{red}$-NADPH complex (Scheme 2) (Seo et al. 2016a, b).

$$\text{FNR}_{red} + \text{NADPH} \rightleftarrows \text{FNR}_{red}\_\text{NADPH}$$
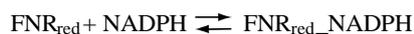
Scheme 2

These results suggest that the hydride transfer process in the reaction of ΔF337 and ΔS338 *Ct*FNRs$_{ox}$ with NADPH is very fast, as is the case for WT *Ct*FNR (Daff 2004; Seo et al. 2016b).

In contrast, NADPH oxidation by ΔY326 *Ct*FNR$_{ox}$ gave rise to a slow absorption change after

the initial rapid drop in the flavin absorption band I (Fig. 3a, b). In phase I of ΔY326 CtFNR$_{ox}$, mixing with NADPH and S-NADPD provided a similar Δabsorbance (Fig. 3b). In this period, the equilibria between FNR$_{ox}$ and CTC-1 (Scheme 1) almost reached completion because the absorbance of the CT band nearly reached its maximum (inset of Fig.3b). Next, k_{obs} values of phase II (k_1 in Table 2) were almost constant between 200-500 μM NADPH (Table 2) and became markedly slower upon deuterium substitution (15 s^{-1} vs. ~200 s^{-1}, kinetic isotope effect value ≈ 13) (Fig. 3b trace b–e). Also, the Δabsorbance of phase II was almost same between 200-500 μM NADPH (ΔA_1 in Table 2). Third, the amplitude of phase III in NADPH oxidation by ΔY326 CtFNR$_{ox}$ increased with increasing NADPH concentration (ΔA_2 in Table 2). Upon mixing with equimolar NADPH, phase III was absent (Fig. 3d; Table 2). Such a NADPH concentration dependence of phase III after formation of MC-1 in the system represented in Scheme 1 can be explained by the formation of the CtFNR$_{red}$_NADPH complex (Scheme 2) (Batie and Kamin 1986, Seo et al. 2016a,b). These results indicate that the k_{obs} of phase II would correspond to the hydride transfer rate, $k_f + k_r$ (Scheme 1) in the case of ΔY326 CtFNR. Accordingly, $k_f + k_r$ can be estimated to be ~200 s^{-1} from k_1 in Table 2 (Daff 2004; Seo et al. 2016a). The smaller $k_f + k_r$ value of ΔY326 CtFNR compared with those of WT, ΔF337 and ΔS338 CtFNRs (>500 s^{-1}) suggests that the residues Tyr326–Val336 contribute to an efficient hydride transfer between NADP$^+$/NADPH and the FAD prosthetic group. It should be noted that although the rate of hydride transfer was significantly decreased in ΔY326 CtFNR, the value is still larger than the maximal velocity of the steady-state reaction assay (~400 s^{-1}/e$^-$ at 10°C (Table 2) vs. 213 s^{-1}/e$^-$ at 25°C (Table 1)), which may be the reason for the slight changes in the steady-state reaction parameters (Table I).

On our measurement timescale, phase IV seems unique to the reaction of ΔY326 CtFNR$_{ox}$ with NADPH. Although the k_r/k_f ratio (Scheme 1) of ΔY326 CtFNR from the equilibrium of phase II (3:7, from $\Delta A_1/(\Delta A_2+\Delta A_3)$ at 100 μM NADPH [Table 2]) was comparable to that of WT CtFNR (3:7, Seo et al. 2016b), the presence of phase IV drastically increased the amount of ΔY326 CtFNR$_{red}$ at equilibrium with NADP$^+$/NADPH compared to WT, ΔF337 and ΔS338 CtFNRs (Fig. 3a, 4a, 5a, Seo et al. 2016b). Based on the transient spectroscopic data and results of the global analyses, we could not assign a reaction step that would corresponded to phase IV. A comparison of the spectra of component B with those of component C and D in the inset of Fig. 3a proves decreases in the absorbance of both the flavin band I and the CT band region. It is likely that the equilibria in Scheme 1 and 2 changed in this phase, and the formation of uncertain species did not provide a substantial unique spectral feature distinctive from other CtFNR$_{red}$-related species.

Previous pre-steady state kinetic studies of the reactions of WT BsFNR and WT CtFNR with NADP$^+$/NADPH confirmed that the reaction involves formation of both CTC-1 (FNR$_{ox}$-NADPH) and CTC-2 (FNR$_{red}$-NADP$^+$), with the appearance of CT absorption bands centered at approximately 600 nm (Blankenhorn 1975; Seo et al. 2016a, b). In this work, ΔF337 and ΔS338 CtFNRs also exhibited a CT absorption band centered at around 600 nm during their reduction by NADPH (Fig. 4a, 5a),

indicating formation of CTC-1 and CTC-2 (Seo et al 2016a, b). For $\mathit{\Delta}$Y326 CtFNR, A_{590} only contained one kinetic component with a corresponding rate constant (~27 s^{-1}) (inset of Fig.3b) similar to that of phase Ⅲ at A_{457} (~30 s^{-1}, k_2 in Table 2), and global analysis provided the spectra of kinetic component B, which had a similar intensity for the CT band compared to that of kinetic component A (in the inset of Fig. 3a). Thus, CTC-2 of $\mathit{\Delta}$Y326 in the NADPH oxidation reaction also possesses a CT absorption band.

For phase Ⅳ, the global analysis indicated that a substantial amount of CTC-1 and CTC-2 remained at equilibrium in $\mathit{\Delta}$F337 and $\mathit{\Delta}$S338 CtFNRs (components C and D in the insets of Fig. 4a, 5a), whereas CTC-1 and CTC-2 are almost omitted in $\mathit{\Delta}$Y326 CtFNR (component D in the inset of Fig.3a). As the redox potential of the free FAD molecule is higher than that of the NADP$^+$/NADPH couple, interactions of FAD with the protein peptide chains are indispensable for regulating redox properties for catalysis. As CtFNR undertakes NADP$^+$ reduction rather than NADPH oxidation under physiological conditions, such regulations might result in a drastic change of the redox properties of the FAD prosthetic group. Our results indicate that the C-terminal region of CtFNR participates in the stabilization of CTCs, thereby regulating the redox properties. It should be noted that the C-terminal region of one protomer covers the re-face of the other protomer in the TrxR-type FNR, which would explain the requirement for the formation of a homodimer as the catalytic unit. Although kinetic analyses in this work can be performed without consideration of the differences in kinetic behavior for each protomer, we cannot exclude the possibility that phase Ⅳ is related to a decreased contact between the protomers due to the truncation.

In reports on flavoproteins of the transhydrogenase family with the two-nucleotide binding domain architecture, the role of the re-face aromatic residue in hydride transfer has often been investigated. In the crystal structure of human GR with NADP$^+$, the re-face Tyr197 residue stacking over the nicotinamide ring portion of NADP$^+$ compresses the nicotinamide ring portion of NADP$^+$ toward the isoalloxazine ring portion of the FAD, resulting in a closer contact between N5 of the isoalloxazine ring portion of FAD and C4 of the nicotinamide ring portion of NADP$^+$, thereby enhancing the hydride transfer (Berkholz et al. 2008). Based on molecular dynamics and quantum mechanics/molecular mechanics simulation of plant-type $Anabaena$ FNR, it has been proposed that the presence of the C-terminal residue Tyr303 on the re-face of the isoalloxazine ring portion of the FAD prosthetic group suppresses the interaction between the isalooxazine ring portion of the FAD hydroquinone anion and the nicotinamide ring portion of NADP$^+$, which contributes to an efficient hydride transfer toward NADP$^+$ (i.e. NADP$^+$ reduction) (Lans et al. 2012; Mulo and Medina 2017).

In the case of the TrxR-type FNR, a motion of the NADPH-binding domain toward FAD-binding domain may allow the positioning of the nicotineamide ring portion of NADP$^+$/NADPH close to the isoalloxazine ring portion of the FAD prosthetic group for hydride transfer (Waksman et al. 1994; Komori et al. 2010). Our result suggests that the C-terminal extension of CtFNR interacts with the

NADPH binding domain thereby stabilizing the conformation of the CTCs. In this process, Phe337 plays a role differently than in the other enzymes. In previous works, replacements of Phe337 also did not significantly affect the steady-state reaction parameters (Muraki et al. 2010). However, it should be noted that any mechanism for the enhancement of hydride transfer involving the C-terminal region of CtFNR might be complicated by the cooperation of other structural factors such as ternary complex formation with Fd (Batie and Kamin 1984). The dynamics of the C-terminal region of CtFNR should be investigated for further understanding of the catalytic details of this protein.

During NADP$^+$ reduction by the WT, $\mathit{\Delta}$Y326 and $\mathit{\Delta}$S338 CtFNRs$_\text{red}$, the k_obs of the faster component of A_{457} decreased with increasing NADP$^+$ concentration (Fig. 3f, 4d). Total absorption changes at 457 nm at 1999 ms demonstrated a significant dependence on NADP$^+$ concentration in the range of 100 to 500 μM (Fig. 3f, 4d). These results indicate that the formation of an FNR$_\text{ox}$-NADP$^+$ complex occurred (Scheme 3), in addition to the reactions in Scheme 1 (Seo et al. 2016a, b). In WT

$$\text{FNR}_\text{ox} + \text{NADP}^+ \rightleftharpoons \text{FNR}_\text{ox} \_\text{NADP}^+$$
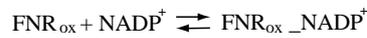
Scheme 3

and both mutated CtFNRs, the k_obs of the fastest component of the change in A_{457} give a similar rate constant of 3–7 s^{-1}. During NADP$^+$ reduction by WT, $\mathit{\Delta}$Y326 and $\mathit{\Delta}$S338 CtFNRs$_\text{red}$, we did not observe a CT band, which makes the determination of the rate-limiting step difficult (Seo et al. 2016b). However, we can conclude that the C-terminal region did not significantly affect the rate-limiting process during NADP$^+$ reduction by CtFNR$_\text{red}$ in the absence of Fd.

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Figure legend

Fig. 1 (a, b) Close-up view around the isoalloxazine ring moiety of the FAD prosthetic group in the crystal structure of CtFNR (PDB code: 3AB1). Main chains of subunits A and B are colored in magenta and aquamarine, respectively. Main chain of the residues 309-348 in subunit B are colored in blue. The side chains of Tyr57 in subunit A, Tyr326 and Phe337 in subunit B are colored in red and depicted as a stick model. The figure was prepared with Discovery Studio 4.0 Visualizer (Accelrys Inc., USA). (c) Partially aligned amino acid sequences of the C-terminal residues in TrxR-type FNRs and EcTrxR. The number of the initial amino acid residue in each line is indicated at left side. The position of the residue stacked on the re-face of the isoalloxazine ring moiety is indicated by an arrow in the upper line. The positions of the 6th and 7th helices assigned in the crystal structure of CtFNR (Muraki et al. 2010) and in EcTrxR (Waksman et al. 1994) are indicated by the waves in the upper and bottom lines, respectively. The positions of C-termini of ΔY326, ΔF337 and ΔS338 CtFNRs are indicated by arrows. bsu:YumC: *Bacillus subtilis subsp. subtilis* str. 168 FNR, rpa:RPA3954: *Rhodopseudomonas palustris* CGA009 FNR, cte:CtFNR: *Chlorobaculum tepidum* FNR, eco:TrxR: *Escherichia coli* K12 TrxR.

Fig. 2 (a) near UV-visible absorption spectra of WT (black broken line), ΔY326 (red continuous line), ΔF337 (green continuous line) and ΔS338 (blue continuous line) CtFNRs in the air-oxidized form. The spectra of approximately 10 μM CtFNRs in 20 mM HEPES-NaOH buffer (pH 7.0) were measured at 23 or 25°C. Absorption coefficient of CtFNR was estimated based on the FAD concentration as described in the Materials and methods section. (b) Thermal stability profiles of air oxidized WT (black circle), $Δ$Y326 (red square), $Δ$F337 (green diamond) and $Δ$S338 (blue triangle) CtFNRs. The supernatant (9-10 μM CtFNR) after incubation at indicated temperature for 5 min followed by centrifugation was retained. Fluorescence intensity of the diluted supernatant (4-5 μM FAD) with excitation at 450 nm and emission at 525 nm was measured. The fluorescence intensities are normalized by subtracting the intensity at 20°C and dividing by the difference intensity between 20°C and 90°C. Each data is average of two or three measurements. (c) Difference spectra of WT (black broken line), ΔY326 (red continuous line), ΔF337 (green continuous line) and ΔS338 (blue continuous line) CtFNRs induced by the addition of 1 mM NADP⁺. Measurements were performed at 23 or 25°C in 20 mM HEPES-NaOH buffer (pH 7.0). (d) Relationship between the magnitude of spectral change and NADP⁺ concentration. Spectral change on the difference spectra of WT CtFNR (black circle): $Δε_{495}$ - $Δε_{514}$, $Δ$Y326 CtFNR (red square): $Δε_{505}$ - $Δε_{477}$, $Δ$F337 CtFNR (green diamond): $Δε_{503}$ - $Δε_{477}$ and $Δ$S338 CtFNR (blue triangle): $Δε_{506}$ - $Δε_{477}$ are plotted against NADP⁺ concentration. Each NADP⁺ solutions were added to the cuvettes containing ~10 μM CtFNR solutions in 20 mM HEPES-NaOH buffer (pH 7.0) for the sample cell and 20 mM HEPES-NaOH buffer (pH 7.0) only for the reference cell.

Fig. 3. (a) Transient spectra induced by mixing 9.3 μM ΔY326 *Ct*FNR$_{ox}$ with 100 μM NADPH. The reaction was performed in 20 mM HEPES-NaOH buffer (pH 7.0) at 10°C. The spectra at 1 ms, 9ms, 99 ms and 999 ms are shown as thin continuous lines. The spectra shown by thin dotted lines from the top to the bottom at 450 nm correspond to those at 2, 19, 49, 199 499, and 1999 ms, respectively. The spectrum of ΔY326 *Ct*FNR$_{ox}$ in the absence of NADPH is shown as a thick continuous line. The arrows indicate the directions of the absorbance changes at the respective wavelengths. The dashed parts of the arrows indicate change that occurred within the first data acquisition period (1 ms). The inset shows the spectrum of each kinetic component resulting from global analysis of the transient spectra with three-step sequential reaction model (A → B → C → D [fast/moderate/slow]). (b) The time course of A_{457} after mixing ΔY326 *Ct*FNR$_{ox}$ with NADPH and *S*-NADPD. The measurement conditions were the same as those in Fig. 3a except the NADPH concentrations of 0 μM (trace a), 100 μM (trace b), 200 μM (trace c) and 500 μM (trace d). The time course of A_{457} after mixing ΔY326 *Ct*FNR$_{ox}$ with 100 μM *S*-NADPD is shown with normalization of *Ct*FNR concentration (trace e). The data are an average of four to five measurements. The red lines indicate fitted curves with two (trace e) or three (trace b-d) exponential components. The residuals are indicated as black lines (NADPH) and a red line (*S*-NADPD) in the lower figure panel. The inset shows time course of A_{590} after mixing ΔY326 *Ct*FNR$_{ox}$ with 100 μM (trace b) and 500 μM (trace d) NADPH. Red lines indicate fitted curves with one exponential component. The residuals are shown as black lines (trace f). (c) Transient spectra induced by mixing 25 μM ΔY326 *Ct*FNR$_{ox}$ with 25 μM NADPH. The reaction was performed in 20 mM HEPES-NaOH buffer (pH 7.0) at 10°C. The spectra at 9, 99 and 999 ms are shown as thin continuous lines. The spectra shown by thin dotted lines from the top to the bottom at 450 nm correspond to those at 4, 19, 49, 199 499, and 1999 ms, respectively. The spectrum of ΔY326 *Ct*FNR$_{ox}$ is shown as a thick continuous line. The arrows indicate the directions of the absorbance changes at the respective wavelengths. The dashed parts of the arrows indicate changes that occurred within the first data acquisition period (1 ms). The inset shows the spectrum of each kinetic component resulting from global analysis of the transient spectra with two-step sequential reaction model (A → B → C [fast/slow]). (d) The time course of A_{457} after mixing 25 μM ΔY326 *Ct*FNR$_{ox}$ with 25 μM NADPH. The measurement conditions were the same as those in Fig. 3c. The data are an average of four measurements. The red line represents a fitted curve with two exponential components. The residual is indicated in the lower figure panel. (e) Transient spectra induced by mixing 10.6 μM ΔY326 *Ct*FNR$_{red}$ with 100 μM NADP$^+$. The reaction was performed in 20 mM HEPES-NaOH buffer (pH 7.0) at 10°C. The spectra at 1 ms, 99 ms and 999 ms are shown as thin continuous lines. The spectra shown by thin dotted lines from the bottom to the top at 450 nm correspond to those at 19, 49, 199, 499 and 1999 ms. The spectrum of ΔY326 *Ct*FNR$_{ox}$ is shown as a thick broken line. The arrows indicate the directions of the absorbance changes at the respective wavelength. The inset shows the spectrum of

each kinetic component resulting from global analysis of the transient spectra with a single-step sequential reaction model [A → B]. (f) The time course of A_{457} after mixing ΔY326 CtFNR$_{red}$ with NADP$^+$. The measurement conditions were the same as those in Fig. 3e, except the NADP$^+$ concentrations of 0 μM (trace a), 100 μM (trace b), 200 μM (trace c) and 500 μM (trace d). The data are an average of four to five measurements. The fitted curves with a function of single exponential component are indicated as red lines. The residuals are indicated with black lines in the lower figure panel.

Fig. 4. (a) Transient spectra induced by mixing 9.3 μM ΔS338 CtFNR$_{ox}$ with 100 μM NADPH. The reaction was performed in 20 mM HEPES-NaOH buffer (pH 7.0) at 10°C. The thin continuous lines from the top to the bottom at 450 nm indicated the spectra at 1 ms, 9 ms, 99 ms, 999 ms and 3999 ms, respectively. The spectra shown by thin dotted lines from the top to the bottom at 450 nm correspond to those at 4 ms, 19 ms, 49 ms, 199 ms, 499 ms and 1999 ms, respectively. The spectrum of ΔS338 CtFNR$_{ox}$ in the absence of NADPH is shown as a thick continuous line. The spectrum of ΔS338 CtFNR$_{red}$ is shown as a thick broken line. The arrows indicate the directions of the absorbance changes at the respective wavelengths. The dashed parts of the arrows indicate change that occurred within the first data acquisition period (1 ms). The inset shows the spectrum of each kinetic component resulting from global analysis of the transient spectra with three-step sequential reaction model (A → B → C → D [fast/moderate/slow]). (b) The time course of A_{457} after mixing ΔS338 CtFNR$_{ox}$ with NADPH and S-NADPD. The measurement conditions were the same as those in Fig. 4a except the NADPH concentrations of 0 μM (trace a), 100 μM (trace b), 200 μM (trace c) and 500 μM (trace d). The data are an average of four to five measurements. The time course of A_{457} after mixing ΔS338 CtFNR$_{ox}$ with 100 μM S-NADPD is shown as trace e with normalized FNR concentration. The fitted curves with a function of two exponential (trace b-d) and three exponential (trace e) components are indicated as red lines. The residuals are indicated as black lines (NADPH) and a red line (S-NADPD) in the lower figure panel. (c) Transient spectra induced by mixing 11.7 μM ΔS338 CtFNR$_{red}$ with 100 μM NADP$^+$. The reaction was performed in 20 mM HEPES-NaOH buffer (pH 7.0) at 10°C. The spectra at 1 ms, 99 ms, 999 ms and 3999 ms are shown as thin continuous lines from the bottom to the top at 450 nm. The spectra shown by thin dotted lines from the bottom to the top at 450 nm correspond to those at 19 ms, 49 ms, 199 ms, 499 ms and 1999 ms, respectively. The spectrum of ΔS338 CtFNR$_{ox}$ is shown as a thick broken line. The arrows indicate the direction of the absorbance change at the respective wavelengths. The inset shows the spectrum of each kinetic component resulting from global analysis of the transient spectra with two-step sequential reaction model (A → B → C [fast/slow]). (d) The time course of A_{457} after mixing ΔS338 CtFNR$_{red}$ with NADP$^+$. The measurement conditions were the same as those in Fig. 4c except the NADP$^+$ concentrations of 0 μM (trace a), 100 μM (trace b), 200 μM (trace c) and 500 μM (trace d). The data are an average of four to five measurements. The

fitted curves with a function of two exponential components are indicated as red lines. The residuals are indicated as black lines in the lower figure panel.

Fig. 5 (a) Transient spectra induced by mixing 9.0 μM ΔF337 CtFNR$_{ox}$ with 100 μM NADPH. The reaction was performed in 20 mM HEPES-NaOH buffer (pH 7.0) at 10°C. The thin continuous lines from the top to the bottom at 450 nm indicated the spectra at 1 ms, 9 ms, 99 ms and 999 ms, respectively. The spectra shown by thin dotted lines from the top to the bottom at 450 nm correspond to those at 4 ms, 19 ms, 49 ms, 199 ms, 499 ms, 1999 ms and 3999 ms, respectively. The spectrum of ΔF337 CtFNR$_{ox}$ in the absence of NADPH is shown as a thick continuous line. The arrows indicate the directions of the absorbance changes at the respective wavelengths. The dashed parts of the arrows indicate changes that occurred within the first data acquisition period (1 ms). The inset shows the spectra of kinetic components resulting from global analysis of the transient spectra with two-step sequential reaction model (A → B → C [fast/slow]). (b) The time course of A_{457} after mixing ΔF337 CtFNR$_{ox}$ with NADPH and S-NADPD. The measurement conditions were the same as those in Fig. 5a except the NADPH concentrations of 0 μM (trace a), 100 μM (trace b), 200 μM (trace c) and 500 μM (trace d). The data are an average of four to five measurements. The time course of A_{457} after mixing ΔF337 CtFNR$_{ox}$ with 100 μM S-NADPD is shown as trace e with normalized FNR concentration. The fitted curves with functions of two exponential (trace b-d) and three exponential (trace e) components are indicated as red lines. The residuals are indicated as black lines (NADPH) and a red line (S-NADPD) in the lower figure panel.

Table 1

Enzymatic, spectroscopic and molecular mass properties of WT, ΔY326, ΔF337 and ΔS338 CtFNRs.

	WT	ΔY326	ΔF337	ΔS338
NADPH diaphorase with ferricyanide[a]				
K_m for NADPH (μM)	14.2 ± 0.9	9.9 ± 0.5	10.2 ± 0.4	10.9 ± 0.6
k_{cat} (s^{-1})	213 ± 3	190 ± 2	161 ± 1.3	172 ± 2
k_{cat}/K_m (×10^6 M^{-1}s^{-1})	15.0 ± 0.2	19.2 ± 0.2	15.8 ± 0.13	15.8 ± 0.18
K_d for NADP$^+$ (μM)[b]	7.6 ± 0.4	12.5 ± 0.4	11.5 ± 1.3	13.6 ± 0.3
ε (mM^{-1}cm^{-1} per subunit) / at λ max (nm)	10.3/ 466[c]	10.3/ 457	10.6 / 457	10.3/ 458
M_r(gel-permeation/ SDS-PAGE)(kDa)	91 / 42	68 / 38	66 / 39	71 / 39

Parameter values with ± one standard deviation are represented.

a: with 2 mM ferricyanide in 20 mM HEPES-NaOH buffer (pH 7.0) at 25°C.

b: obtained with the data in Figure 2c.

c: from Seo and Sakurai (2002)

Table 2

Kinetic constants obtained by the pre-steady-state analysis of the reaction of ΔY326CtFNR$_{ox}$ with NADPH.

[NADPH] (μM)	100[a]	200[a]	300	500[a]	25[b]
k_1 (s^{-1})	154 ± 3 (100 ± 15)	208 ± 5 (137 ± 23)	186 ± 5 (123 ± 17)	205 ± 5 (140 ± 30)	117 ± 1.8 (99 ± 10)
ΔA_1	0.0170 ± 0.0002	0.0140 ± 0.0002	0.0146 ± 0.0002	0.0124 ± 0.00017	0.0468 ± 0.0004
k_2 (s^{-1})	25.2 ± 0.3 (18.5 ± 1.6)	29.6 ± 0.2 (26.7 ± 1.3)	28.5 ± 0.3 (24 ± 1.6)	28.7 ± 0.19 (27 ± 1.8)	-
ΔA_2	0.0237 ± 0.0002	0.0277 ± 0.0001	0.0301 ± 0.0002	0.0322 ± 0.00013	-
k_3 (s^{-1})	2.95 ± 0.02 (2.6 ± 0.3)	3.47 ± 0.02 (3.6 ± 0.13)	3.51 ± 0.02 (3.3 ± 0.2)	3.53 ± 0.019 (3.6 ± 0.17)	3.06 ± 0.01 (3.2 ± 0.2)
ΔA_3	0.01815 ± 0.00006	0.01834 ± 0.00006	0.01832 ± 0.00007	0.01797 ± 0.00006	0.07764 ± 0.00009

Rate constants (k) and amplitudes (ΔA) of each kinetic component obtained by fitting with bi- or tri-exponential decay functions against absorption changes at 457 nm at 0-1000 ms time period are represented in the order from the fastest to the slowest. Measurement conditions are described in legends of Fig. 3b and 3d. The value of each parameter is represented with $\pm$ one standard deviation. The rate constant values in the parentheses are those obtained by global analysis of the transition of absorptions with a three-step reaction model (A→B→C→D [fast/moderate/slow]) for 100-500 μM NADPH or a two-step reaction model (A→B→C [fast/slow]) for 25 μM NADPH.

a: mixing with 9.3 μM ΔY326 CtFNR$_{ox}$ with NADPH. Data in Fig.3b were utilized for the estimation.

b: mixing with 25 μM ΔY326 CtFNR$_{ox}$ with 25 μM NADPH. Data in Fig.3d were utilized for the estimation.

Table 3

Kinetic constants obtained by the pre-steady-state analysis of the reaction of ΔS338 *Ct*FNR$_{ox}$ with NADPH.

[NADPH] (μM)	100[a]	200[a]	300	500[a]
k_1 (s^{-1})	50.0 ± 0.4	43.4 ± 0.3	37.5 ± 0.2	36.2 ± 0.4
	$(810 \pm 80, 47 \pm 6)$	$(530 \pm 90, 37 \pm 7)$	$(270 \pm 70, 35 \pm 3)$	$(360 \pm 80, 30 \pm 2)$
ΔA_1	0.01570 ± 0.00008	0.01777 ± 0.00006	0.02116 ± 0.0006	0.0209 ± 0.0001
k_2 (s^{-1})	$1.34 \pm 0.04 \ (1.1 \pm 0.5)$	$1.01 \pm 0.03 \ (1.1 \pm 0.17)$	$0.82 \pm 0.03 \ (0.68 \pm 0.09)$	$1.55 \pm 0.06 \ (0.4 \pm 0.5)$
ΔA_2	0.00637 ± 0.00008	0.0084 ± 0.00014	0.0106 ± 0.0003	0.00788 ± 0.00009

Rate constants (k) and amplitudes (ΔA) of each kinetic component obtained by fitting with bi-exponential decay functions against absorption changes at 457 nm at 0 ms − 1000 ms time region are represented in the order from the fastest to the slowest. Measurement conditions are described in legends of Fig.4b. The value of each parameter is represented with ± one standard deviation. The rate constant values in the parentheses are those obtained by global analysis of the transition of absorptions with a three-step reaction model (A→B→C→D [fast/moderate/slow]). The rate constants of fastest and middle phase are indicated in the same parentheses in k_1 column. Slowest one is indicated in k_2 column.

a: mixing with 9.3 μM ΔS338*Ct*FNR$_{ox}$ with NADPH. Data in Fig.4b were utilized for the estimation.

Table 4

Kinetic constants obtained by the pre-steady-state analysis of the reaction of ΔF337 CtFNR$_{ox}$ with NADPH.

[NADPH] (µM)	100[a]	200[a]	500[a]
k_1 (s^{-1})	41.8 ± 0.4 (50 ± 3)	37.5 ± 0.3 (42 ± 1.8)	30.5 ± 0.16 (30 ± 1.2)
ΔA_1	0.0177 ± 0.0001	0.02083 ± 0.00007	0.02410 ± 0.00006
k_2 (s^{-1})	1.69 ± 0.07 (2.2 ± 0.13)	1.39 ± 0.05 (1.6 ± 0.15)	0.98 ± 0.04 (0.69 ± 0.09)
ΔA_2	0.00555 ± 0.00007	0.00627 ± 0.00008	0.0080 ± 0.00018

Rate constants (k) and amplitudes (ΔA) of each kinetic component obtained by fitting with a bi-exponential decay functions against absorption changes at 457 nm are represented in the order from the fastest to the slowest. Measurement conditions are described in legends of Fig.5b. The value of each parameter is represented with ± one standard deviation. The rate constant values in the parentheses are those obtained from global analysis of the transition of absorptions with a two-step reaction model (A→B→C [fast/slow]).

a: mixing with 9.0 µM ΔF337 CtFNR$_{ox}$ with NADPH. Data in Figure 5b were utilized for the estimation.
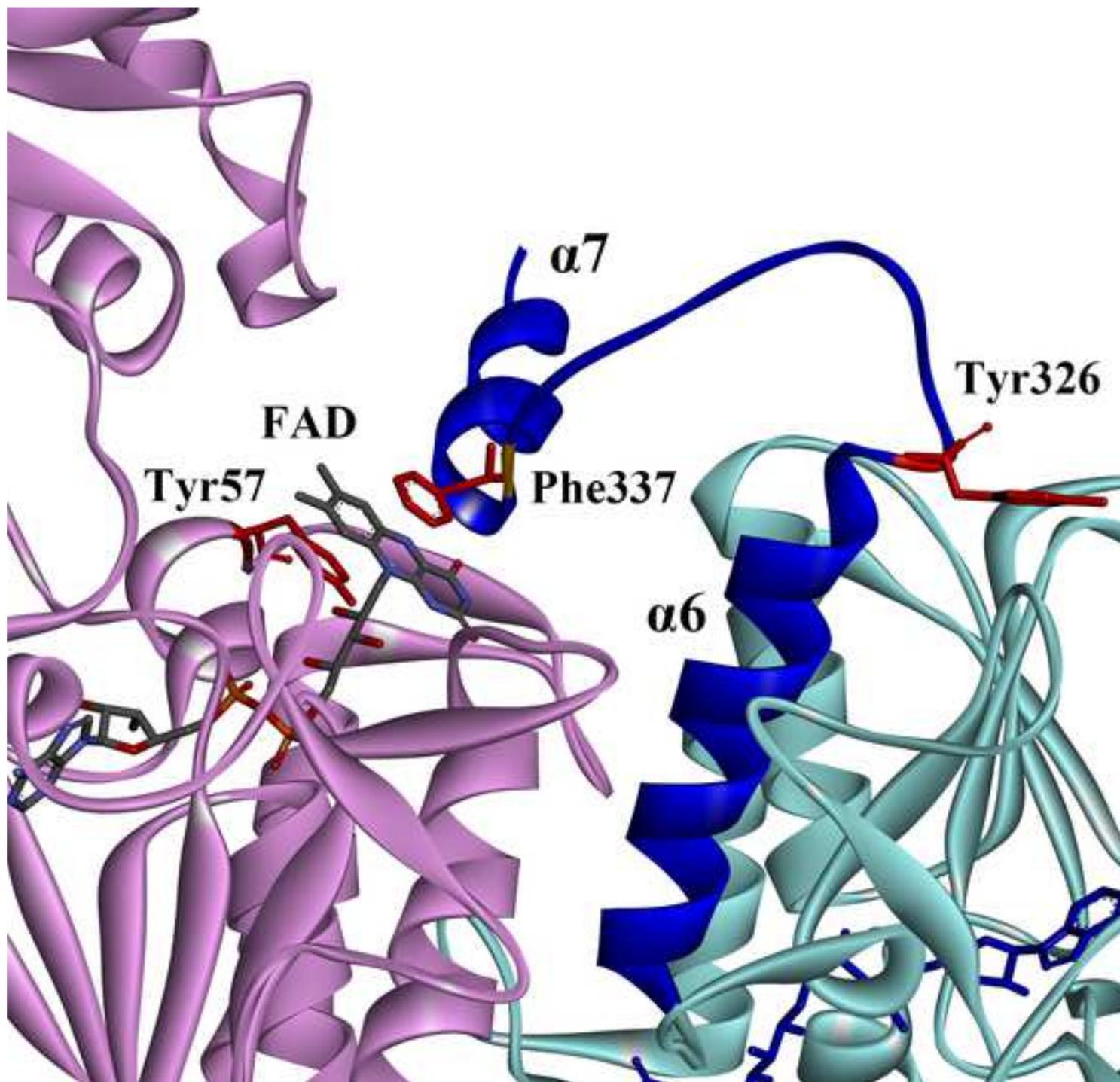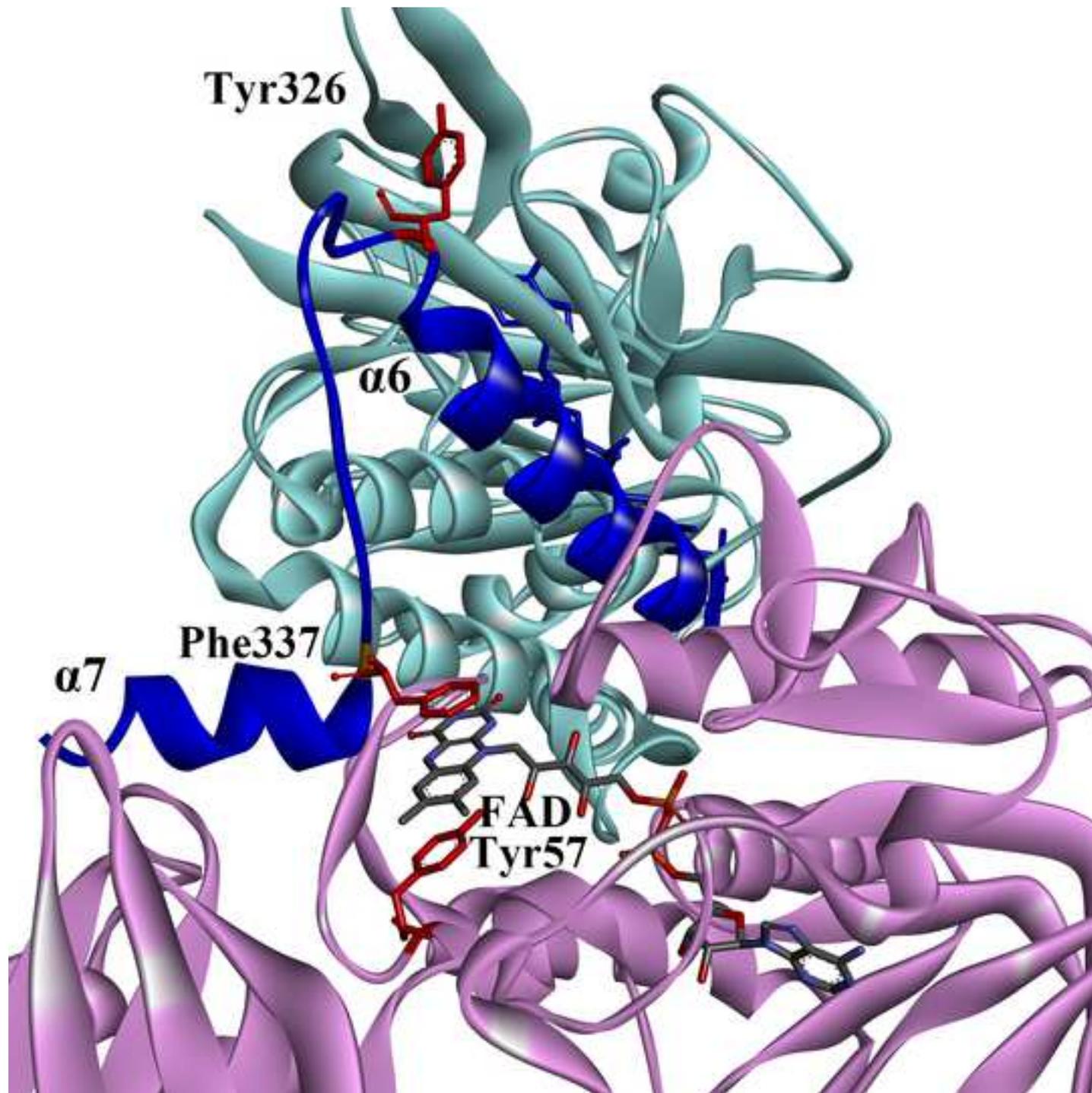
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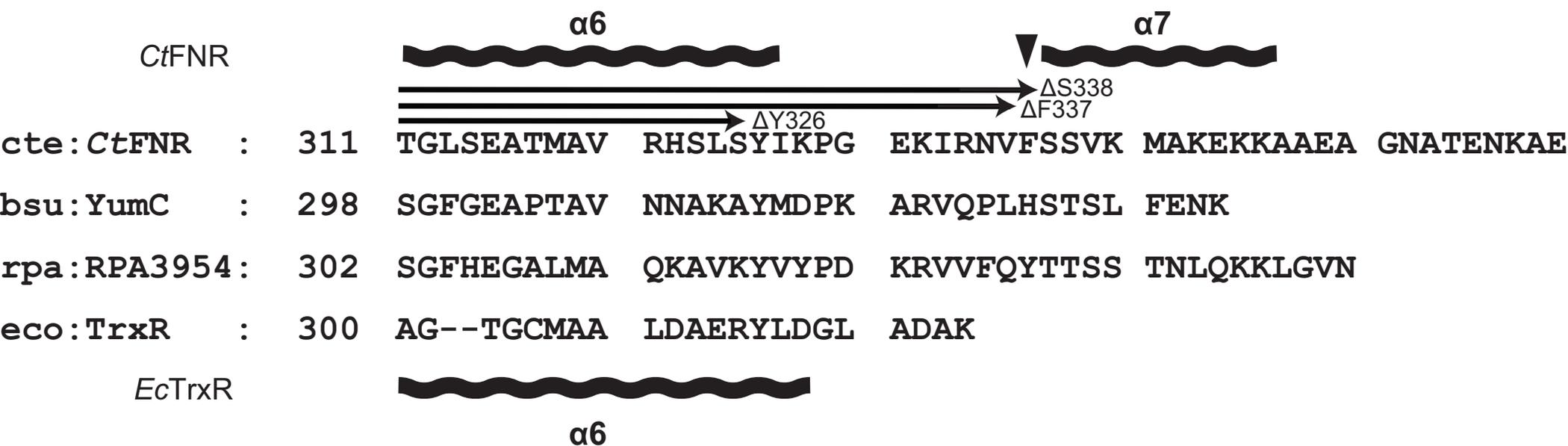
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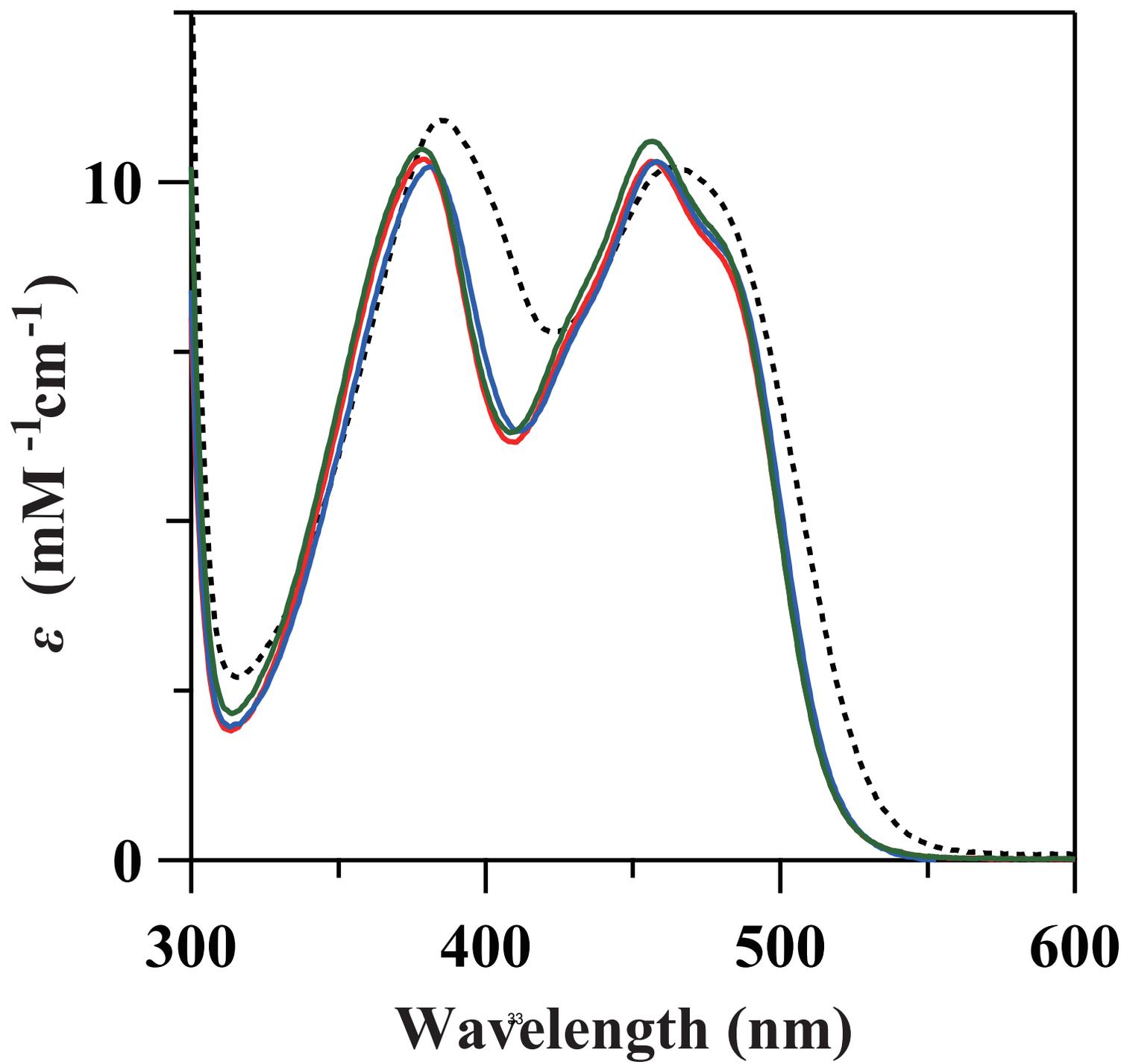
Figure 2b

Figure 2c

Figure 2d

$\varDelta\varepsilon$ (mM^{-1}cm^{-1})

[NADP$^+$] (μM)

Figure 3a

Wavelength (nm)
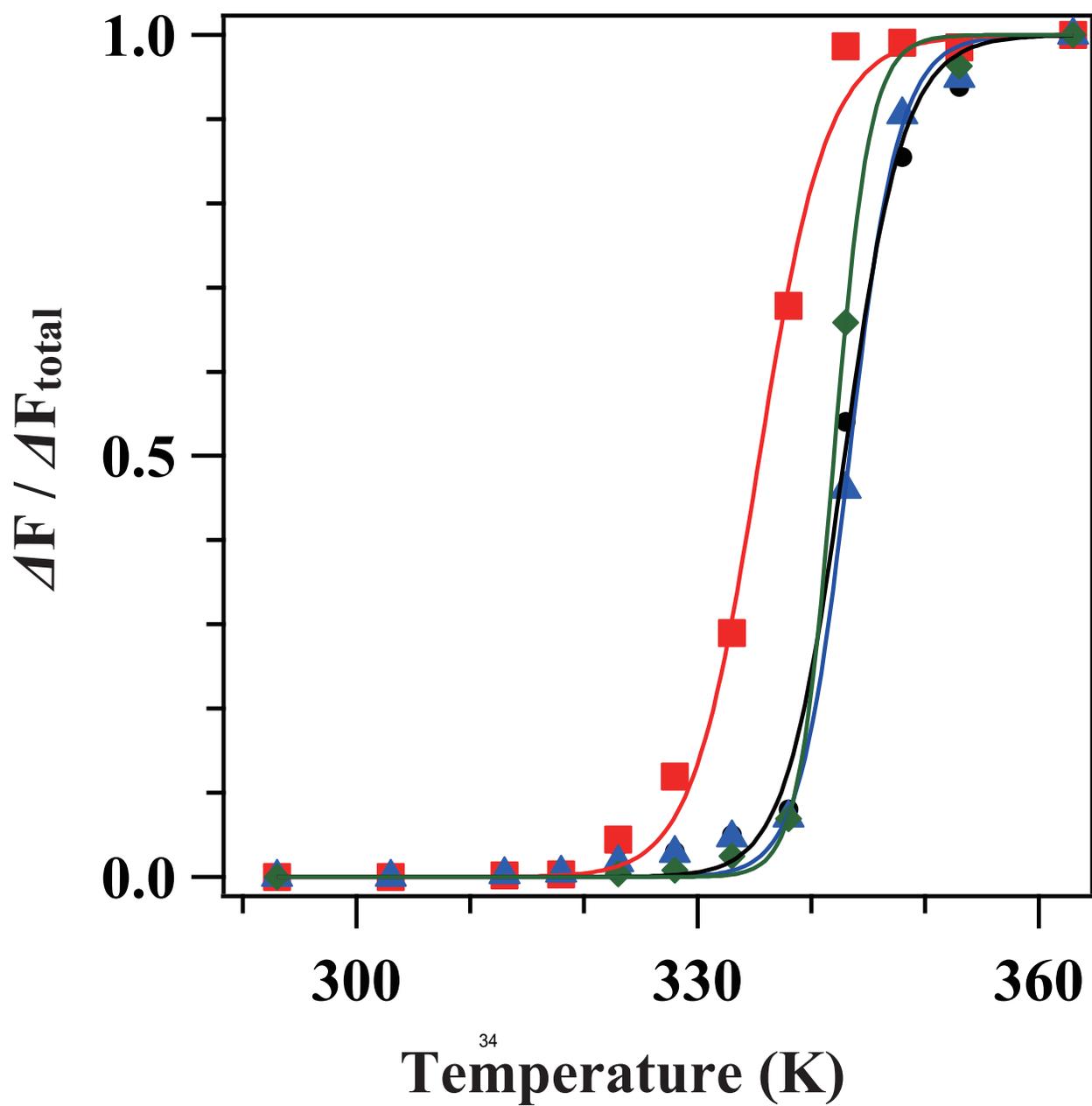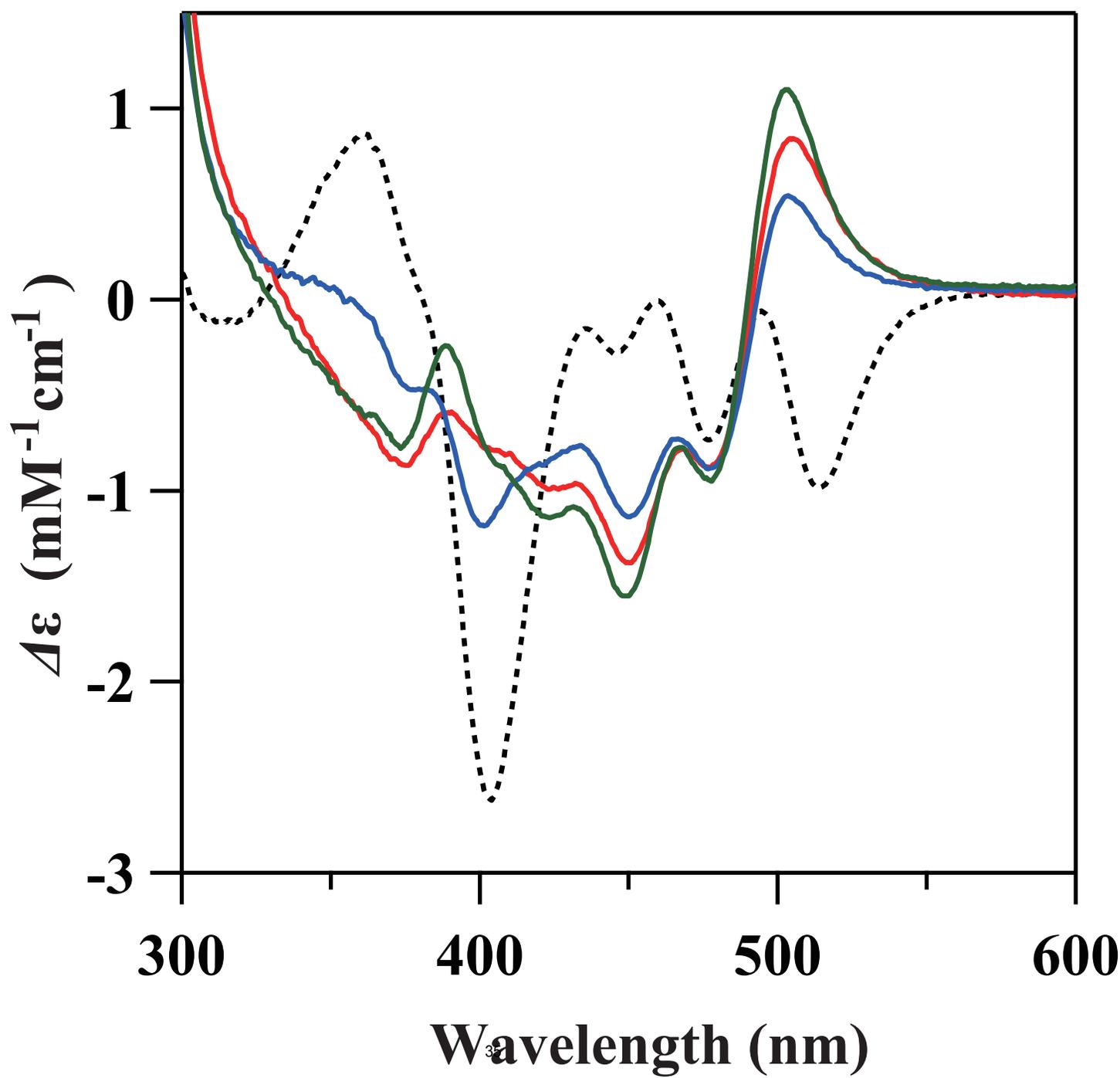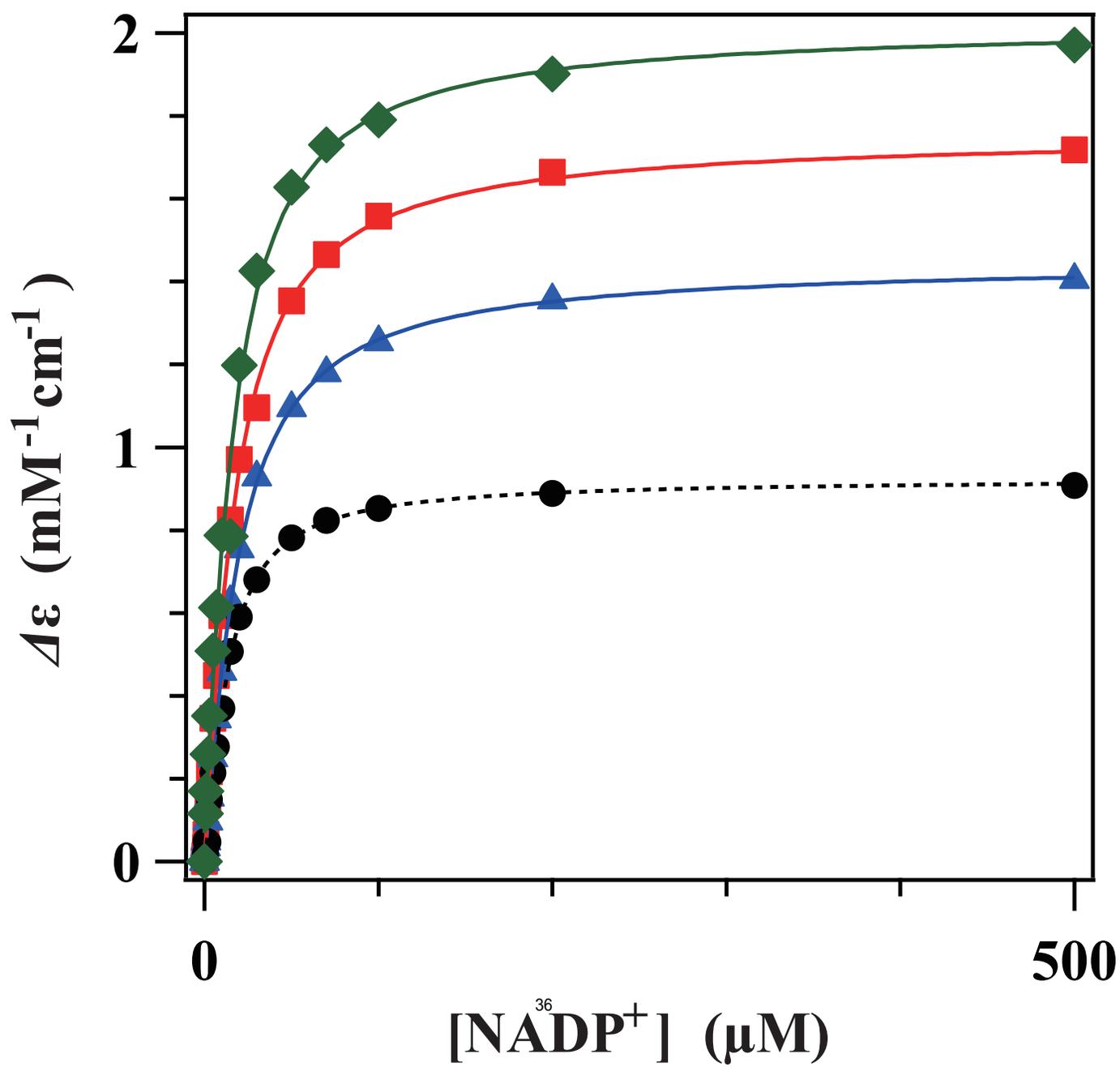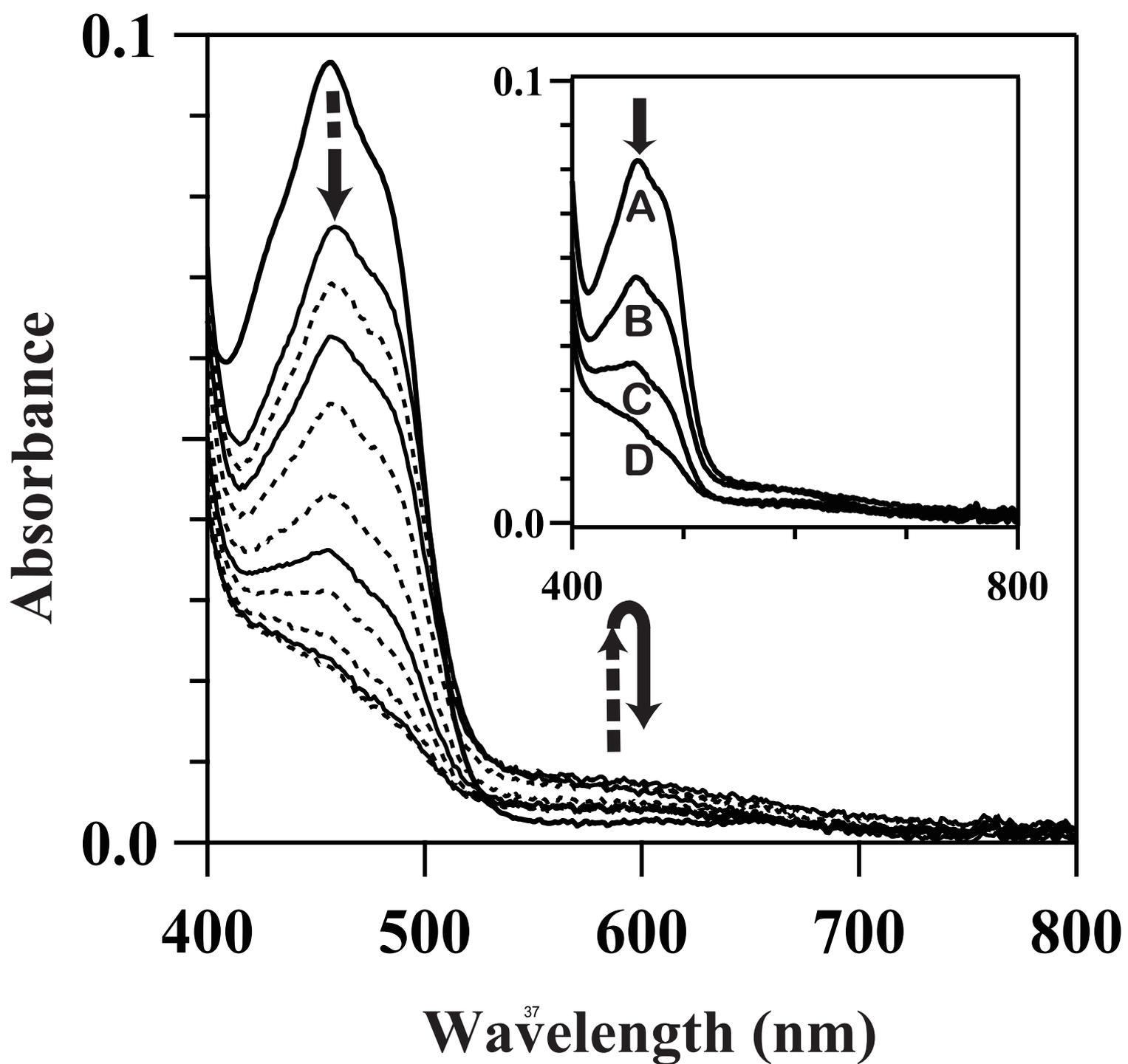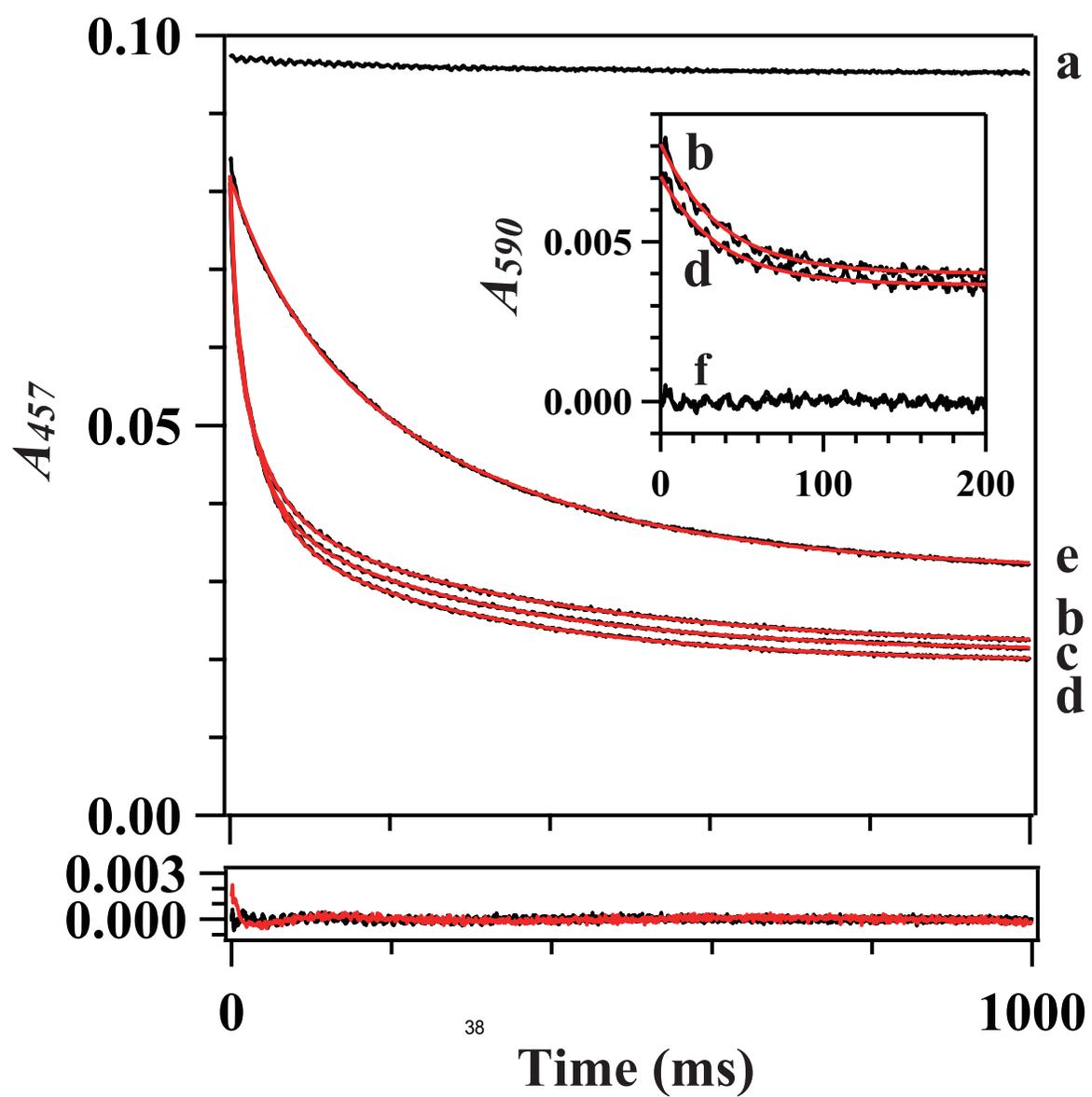
Figure 3b

Figure 3c

Figure 3d

A_{457}

Time (ms)
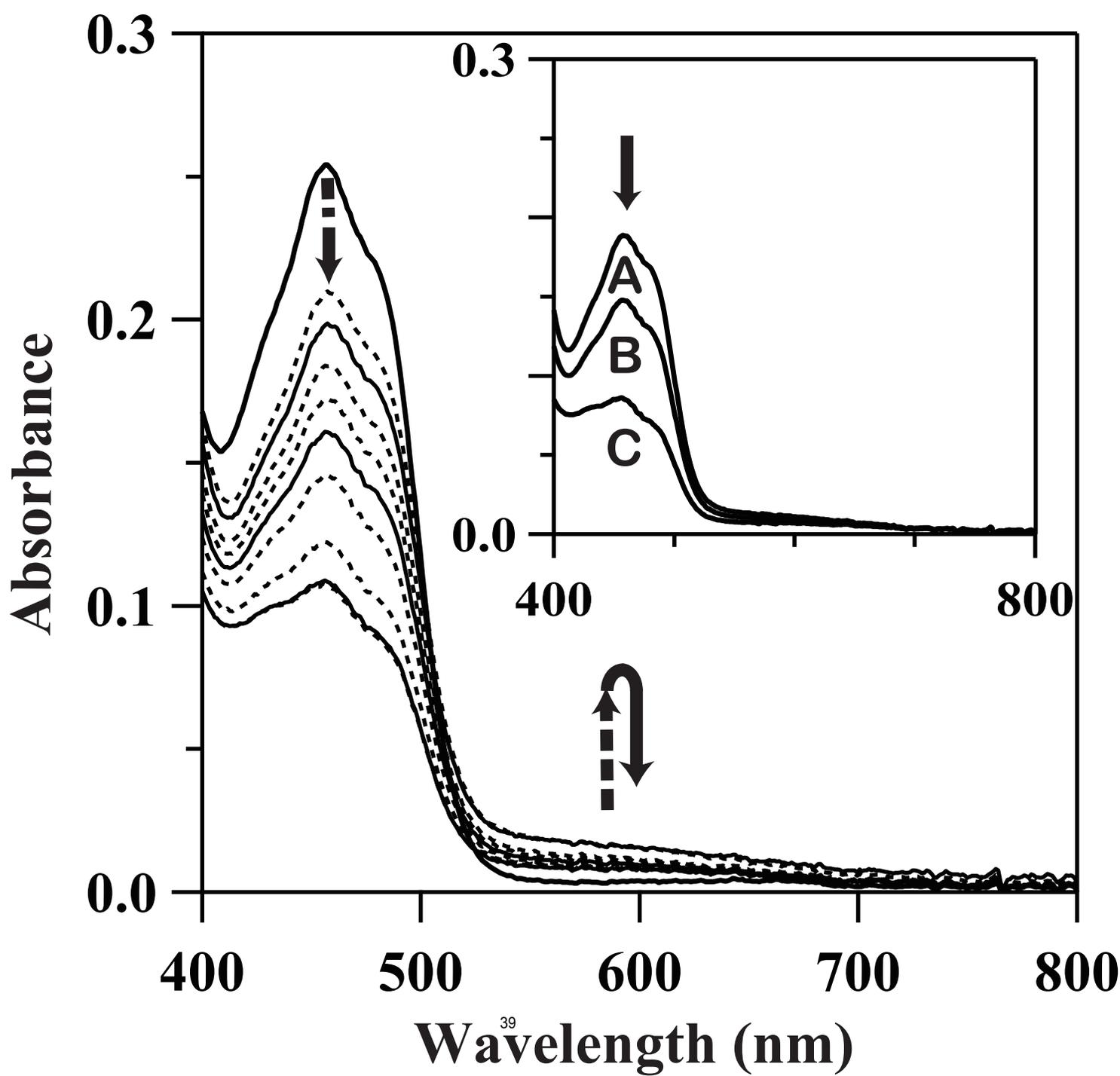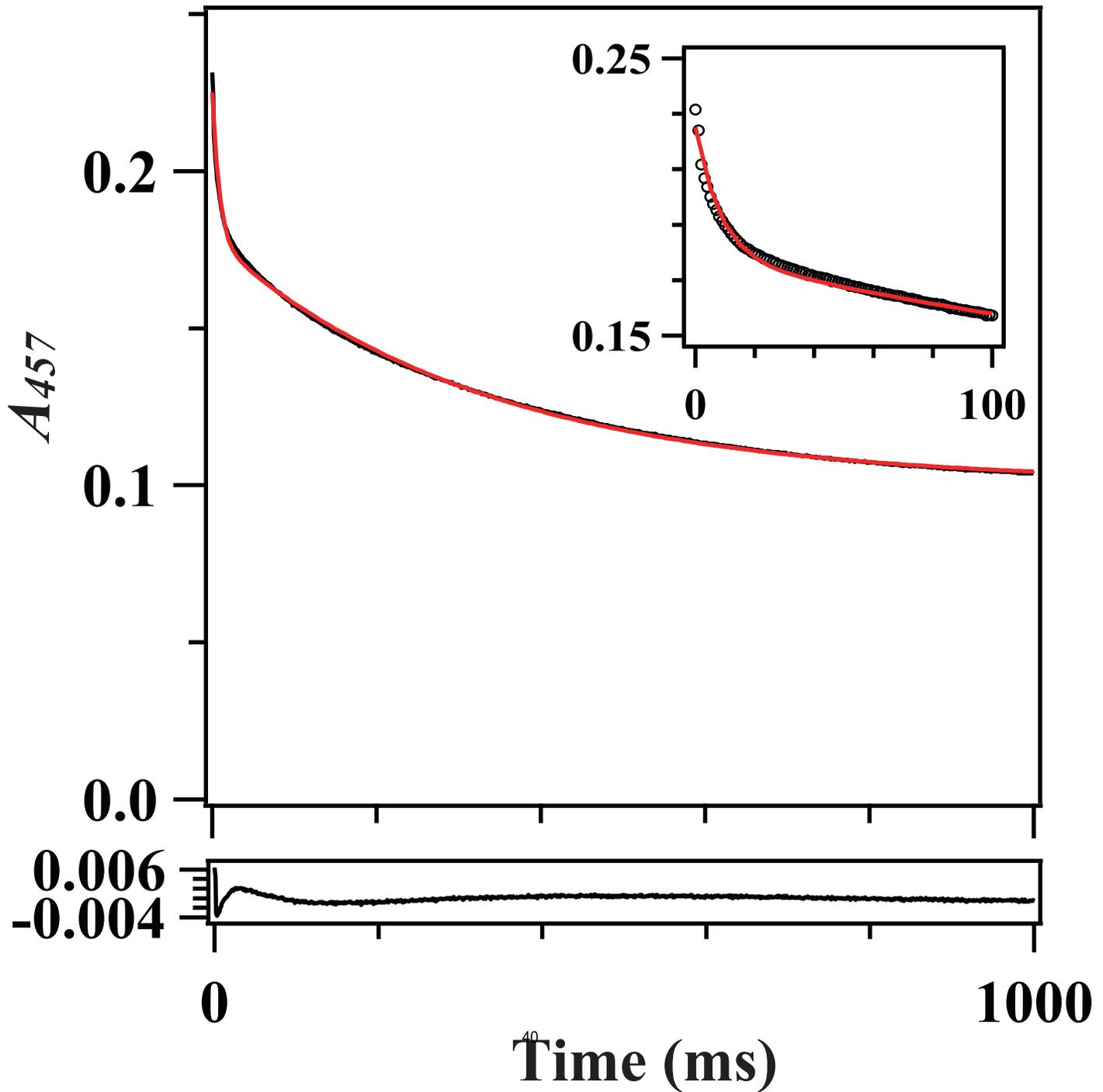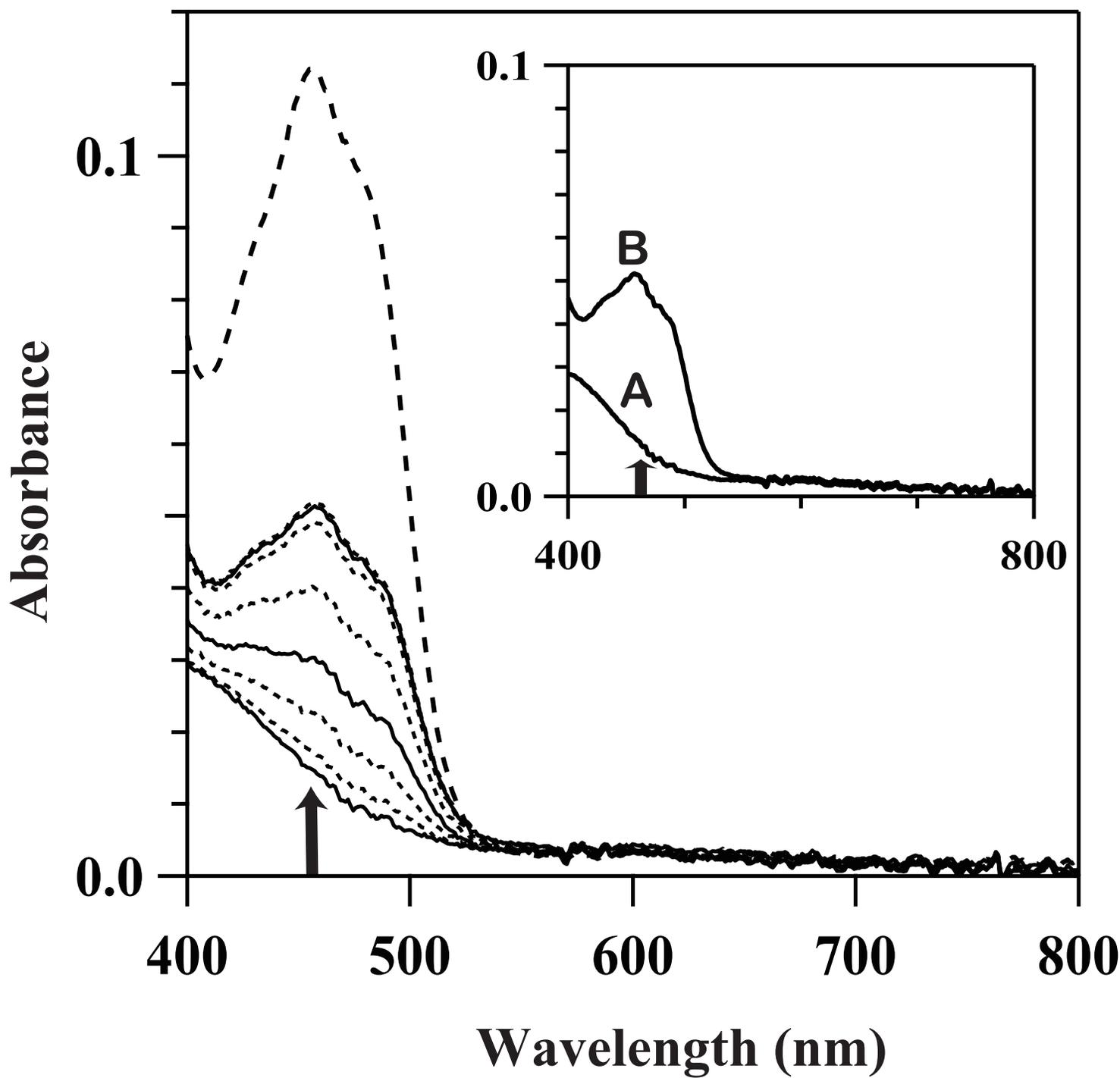
Figure 3e

Figure 3f

Figure 4a

Figure 4b

Figure 4c

Figure 4d

Figure 5a

Figure 5b