Interactions with the Bifunctional Interface of the Transcriptional Coactivator DCoH1 Are Kinetically Regulated*

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Background: The protein dimerization cofactor of HNF-1 (DCoH1)/pterin-carbinolamine dehydratase (PCD) accomplishes two unrelated activities, forming two complexes with the same interface.

Results: The DCoH1 homotetramer is kinetically trapped; a single mutation in the interface increases the unfolding rate 109-fold.

Conclusion: Kinetic regulation allows DCoH to assume two unrelated functions.

Significance: Mutations excluding water from interfaces represent kinetic “hot spots,” dramatically affecting dissociation rates.

PCD is known as DCoH or dimerization cofactor of the transcription factor HNF-1. These two activities are associated with a change in oligomeric state: from two dimers interacting as an enzyme in the cytoplasm to a dimer interacting with a dimer of HNF-1 in the nucleus. The same interface of DCoH forms both complexes. To determine how DCoH partitions between its two functions, we studied the folding and stability of the DCoH homotetramer. We show that the DCoH1 homotetramer is kinetically trapped, meaning once it forms it will not dissociate to interact with HNF-1. In contrast, DCoH2, a paralog of DCoH1, unfolds within hours. A simple mutation in the interface of DCoH2 from Ser-51 to Thr, as found in DCoH1, increases the kinetic stability by 9 orders of magnitude, to 108-fold. This suggests that the DCoH1-HNF-1 complex must cofold to interact. We conclude that simple mutations can dramatically affect the dissociation kinetics of a complex. Residue 51 represents a “kinetic hot spot” instead of a “thermodynamic hot spot.” Kinetic regulation allows PCD to adopt two distinct functions.

Mutations in DCoH1 associated with diabetes affect both functions of DCoH1, perhaps by disrupting the balance between the two DCoH complexes.

Pterin-4a-carbinolamine dehydratase (PCD) is a highly conserved enzyme that evolved a second, unrelated function in mammals, as a transcriptional coactivator. As a coactivator, PCD is known as DCoH or dimerization cofactor of the transcription factor HNF-1. These two activities are associated with a change in oligomeric state: from two dimers interacting as an enzyme in the cytoplasm to a dimer interacting with a dimer of HNF-1 in the nucleus. The same interface of DCoH forms both complexes. To determine how DCoH partitions between its two functions, we studied the folding and stability of the DCoH homotetramer. We show that the DCoH1 homotetramer is kinetically trapped, meaning once it forms it will not dissociate to interact with HNF-1. In contrast, DCoH2, a paralog of DCoH1, unfolds within hours. A simple mutation in the interface of DCoH2 from Ser-51 to Thr, as found in DCoH1, increases the kinetic stability by 9 orders of magnitude, to 108-fold. This suggests that the DCoH1-HNF-1 complex must cofold to interact. We conclude that simple mutations can dramatically affect the dissociation kinetics of a complex. Residue 51 represents a “kinetic hot spot” instead of a “thermodynamic hot spot.” Kinetic regulation allows PCD to adopt two distinct functions. Mutations in DCoH1 associated with diabetes affect both functions of DCoH1, perhaps by disrupting the balance between the two DCoH complexes.

In vertebrates, PCD is one of two enzymes that recycle the tetrahydrobiopterin cofactor of the three aromatic amino acid hydroxylases: phenylalanine hydroxylase generating tyrosine; tryptophan hydroxylase generating the precursor of the neurotransmitter serotonin; and tyrosine hydroxylase generating L-DOPA, the precursor of the neurotransmitter dopamine and the pigment melanin (5–8). In the aromatic amino acid hydroxylation reactions, tetrahydrobiopterin donates two electrons to reduce a catalytic iron and cleave molecular oxygen (7, 9). The first step of tetrahydrobiopterin recycling is catalyzed by PCD, dehydrating the oxidized tetrahydrobiopterin-4a-carbinolamine (10, 11). Tetrahydrobiopterin is also a cofactor of nitric oxide synthase and the membrane-bound glycoprotein erythroid monooxygenase (12, 13). The enzymatic activity of PCD is cytoplasmic, although plant PCD is mitochondrial (4). In angiosperms, yeast, and some bacteria that lack tetrahydrobiopterin, PCD may recycle molybdopterin or other pterin carbinolamines (4).

In mammals, PCD has adopted a second role as a coactivator, interacting with the dimerization domain of the transcription factor HNF-1 (10, 14). As a coactivator, the protein was named DCoH, or dimerization cofactor of HNF-1 (10). There is no known relationship between the enzymatic and coactivator functions of PCD/DCoH (15). Interaction with HNF-1 promotes nuclear localization of DCoH (16, 17). DCoH interacts with the dimerization domains of both HNF-1α and HNF-1β, enhancing HNF-1 transcription either by stabilizing the HNF-1 dimer or by bridging HNF-1 to other coactivators (14).

There is evidence that PCD participates in other as yet undefined interactions. In neurons, PCD is nuclear in the absence of HNF-1, suggesting another partner protein (16–18). DCoH is also a maternal factor and is localized to the nucleus early in embryogenesis (17). Catalytically inactive PCD paralogs exist in plant plastids and photosynthetic bacteria that may function as ribulose-bisphosphate carboxylase/oxygenase (RuBisCo) chaperones (4, 19).

The prokaryotic and archaeal PCDs are obligate dimers (see Ref. 19 and Protein Data Bank (PDB) ID 3jst, 2ebb, and 1uso). Mammalian PCD/DCoH forms a dimer of dimers (20). The same interface of the DCoH dimer that forms the homote-
tramer also interacts with HNF-1 (21). To understand how DCoH cycles between the homotetramer and the heterotetramer with HNF-1, we studied the folding and stability of the DCoH homotetramer (22).

Mammals and birds express two isoforms of DCoH, DCoH1 and DCoH2 (1). In mammals, both isoforms retain enzymatic and catalytic functions. Both are expressed with HNF-1 in hepatocytes, kidney, gut, and pancreas (16, 23, 24). Among cells lacking HNF-1, DCoH1 is expressed in skin, brain, heart, and eye (14, 18, 25), and DCoH2 is expressed in muscle (26). Both DCoH1 and DCoH2 form homotetramers in solution. The major difference in activity between the two DCoH isomers noted in vitro is their ease of interaction with HNF-1; the DCoH2/HNF-1 heterodimer forms after mixing DCoH2 homotetramers with HNF-1 dimers, whereas the DCoH1 homotetramer only associates with HNF-1 if they co-fold together (1, 21, 27, 28). This suggested that the DCoH1 homotetramer is thermodynamically more stable than the DCoH1/HNF-1 heterotetramer. We previously showed that refolding and unfolding of DCoH1 by guanidinium chloride (GdnHCl) exhibited hysteresis, with an unfolding midpoint \( C_{m} \) of 3 M and refolding \( C_{m} \) of 1.25 M (22). DCoH2 unfolding displayed no such hysteresis. The tetramerization interface between DCoH1 and DCoH2 is highly conserved, except for amino acid 51 at the center of the interface that is a Thr in DCoH1 and a Ser in DCoH2. Four copies of residue 51 interact at the center of the tetramer, one from each monomer. The Thr to Ser mutation in DCoH1 eliminated the hysteresis. To explain the hysteresis, we proposed that the DCoH1 homotetramer is not thermodynamically stable but kinetically trapped.

The current study is the first to report the unfolding kinetics of the DCoH homotetramer. By mutating Ser-51 of DCoH2 to Thr, as found in DCoH1, we introduce folding hysteresis to DCoH2. Kinetic measurements confirm that the homotramer with Thr-51 unfolds very slowly. We propose that residue 51 represents a “kinetic hot spot” within the DCoH tetramer interface. Kinetic regulation of the DCoH1 homotetramer implies that the coactivator and enzymatic pools of DCoH1 are distinct and do not interconvert.

### EXPERIMENTAL PROCEDURES

**Cloning, Protein Expression, and Purification—Mouse DCoH2** was subcloned into the BamHI and EcoRI restriction sites of a pET-24b vector (Novagen) modified with an N-terminal His tag followed by a thrombin cleavage site. To prevent formation of a disulfide bond during purification, the conserved residue Cys-82 was mutated to Ser (1). Mutagenesis was accomplished using the PCR-based procedure described with the Phusion® high-fidelity DNA polymerase, with the forward primer: ACC TCG CAT GAC TTC GCC GGC CTT ACC AAG (mutated codon underlined, changed from TGT), and the reverse primer: GAG GGT TAT CTT GAC TTT GTG GTA GAC GTT GAA. Constructs in this study all include the C82S mutation, unless otherwise stated.

The DCoH2 S51T mutant was generated with the forward primer: A GCA GAG AAG ATG AAT CAC CAC CCG GAA TG, and the reverse primer: TG CAG GCC AAC CCT GGT CAT AAA GCC AAA C. The DCoH2 S51Q mutant was generated with the reverse primer: TG CAG GCC AAC CCT CTG CAT AAA GCC AAA C.

The DCoH2 S51T gene was overexpressed in *Escherichia coli* strain BL21 Star (DE3) (Invitrogen) grown at 37 °C to \( A_{600} \) of 0.6 – 0.9, and induced with 0.5 mM isopropyl-1-thio-\( \beta \)-D-galactopyranoside (Gold Biotechnology®) for 5 h. For purification of DCoH2 S51T, cell pellets were resuspended in pre-chilled lysis buffer: PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.3), plus 300 mM NaCl, and 10 mM imidazole. Cells were lysed by sonication followed by centrifugation to remove cell debris. His-DCoH2 was isolated through batch purification using His-Select® nickel affinity gel (Sigma), according to the manufacturer’s protocol. The eluted protein was dialyzed into PBS (pH 7.3) plus 300 mM NaCl. The His tag was cleaved by incubating with 1 unit of thrombin (MP Biomedicals) per mg of protein at 4 °C for 2 days. The His tag was removed by dialysis or low-speed centrifugation at 4 °C in an Amicon centrifugal filter unit (molecular weight cut-off of 30,000). Purified DCoH2 was concentrated to 2 mM as determined by UV absorbance using the calculated extinction coefficient of 19480 M\(^{-1}\) cm\(^{-1}\) (29). Monomer concentrations are reported throughout the study. The protein was greater than 95% pure, as detected by Coomassie Blue-stained SDS-PAGE gels. DCoH2 S51Q was purified with a similar protocol except with 700 mM NaCl added to the lysis and dialysis buffers to prevent precipitation.

**Crystallization and Structure Determination**—Crystallization conditions of DCoH2 S51T (12.85 mg/ml) were identified with Crystal Screen 1 (Hampton Research) by the sitting drop method. The final optimized crystal conditions were: 0.1 M Tris buffer (pH 7.5), and 8% polyethylene glycol 8,000 (PEG 8,000, Hampton Research) at 18 °C. Crystals were soaked in reservoir solution plus 20 or 30% PEG 400 (Hampton Research) as a cryoprotectant and flash-frozen in liquid nitrogen. Data were collected at 100 K on the Mar 225 CCD detector at the Southeast Regional Collaborative Access Team (SER-CAT) beamline 22-BM at the Advanced Photon Source (APS, Argonne National Laboratory, Argonne, IL). 180 frames were collected of 1° oscillations at a crystal to detector distance of 120 mm. Data collection statistics are listed in Table 1. The data were processed with HKL-2000 and Scalepack (HKL Research Inc.).

Initial phases were determined by molecular replacement with the CCP4 program Phaser (30) using the 1.6 Å wild-type DCoH2 structure (PDB ID: 1RU0) as a search model (1). The search model consisted of a DCoH2 dimer with the Ser-51 residue replaced by Ala and all water molecules removed. The model was refined with PHENIX including rigid body refinement, individual atomic coordinates, individual atomic displacement parameters, and automatic solvent placement (31). Manual building was carried out with Coot (32). Final refinement statistics are reported in Table 1.

**Equilibrium Unfolding and Refolding**—Equilibrium unfolding and refolding of DCoH2 proteins were monitored at varying GdnHCl (Pierce) concentrations through changes in intrinsic tryptophan fluorescence using a PTI C-61 spectrofluorometer (Photon Technology International, Birmingham, NJ) as de-
scribed in Ref. 22. Data were collected at room temperature (25 °C) from 300 to 400 nm at a scan rate of 1 nm/s. The average emission wavelength (AEW) was calculated with the equation

\[ \text{AEW} = \frac{\sum(I_i \times \lambda_i)}{\sum I_i} \]  

(Eq. 1)

where \( I_i \) is the absolute fluorescence signal at the wavelength of \( \lambda_i \), with the baseline subtracted.

Baselines were measured at each unfolding/refolding condition in the absence of protein. AEW data were plotted as a function of GdnHCl concentration with Excel (Microsoft).

Unfolding samples were diluted to a series of GdnHCl concentrations in PBS and incubated at room temperature for over 20 h in 2-ml low-retention microcentrifuge tubes (Fisher Scientific) to minimize protein loss, as described in Ref. 22. The GdnHCl concentration was confirmed by refractive index with a refractometer (Fisher Scientific). Equilibrium unfolding of 1.6, 4.8, and 16 mM DCoH2 C82S was similar to wild-type DCoH2 (wtDCoH2); confirming that the C82S mutation did not alter the stability of DCoH2 (not shown). All folding reactions were replicated in triplicate to obtain average values and standard deviations.

For refolding measurements, samples were unfolded by incubating at 6.5 mM GdnHCl in PBS for DCoH2 S51T or in 4 mM GdnHCl for DCoH2 S51Q, at room temperature for at least 12 h. The samples were then diluted to various GdnHCl concentrations in PBS and incubated for another 12 h. Fluorescence signals were measured for both protein samples and baseline samples.

Equilibrium unfolding was measured by CD using a PiStar spectrophotometer (Applied Photophysics) at a wavelength of 225 nm for 30 s. For samples measured by both fluorescence and CD, fluorescence was measured first followed by CD.

For CD measurements, 10-ml unfolding samples were prepared for each GdnHCl concentration and monitored daily. For fluorescence measurements, 40-ml samples were prepared at each GdnHCl concentration and monitored for 24 h. At each time point, 1 ml was removed from the stock for a fluorescence average emission wavelength scan, as described above. Unfolding kinetics were measured by CD at 225 nm over time with 1,000 time points recorded. Samples were measured only once to minimize sample damage. 2-ml baseline samples were prepared for each GdnHCl concentration and measured at time 0 and 24 h.

For fluorescence measurements of DCoH2 S51T, 40-ml samples were prepared at each GdnHCl concentration and monitored for 9 days as above. 10-ml baseline control samples were prepared for each GdnHCl concentration and monitored daily. For CD measurements, 10-ml unfolding samples were prepared at each GdnHCl concentration. To minimize evaporation, 1-ml samples were measured continuously for 24 h and replaced by fresh samples from the stock reactions. Samples were also sealed in a quartz cuvette with Parafilm.

DCoH2 S51Q unfolded quickly, within minutes. Samples were mixed by hand; therefore only GdnHCl concentrations near the \( C_{u}\) of \( \approx 1.5 \) M GdnHCl (see Fig. 1) were slow enough to measure on the CD spectrometer. For refolding, samples were unfolded with 4 M GdnHCl for 20 h and then diluted to 1.67 or 2.07 M GdnHCl.

Curve Fitting—Equilibrium data were normalized and plotted as fraction folded as described in Ref. 22. All equilibrium unfolding and refolding curves appeared to be single transitions as measured by fluorescence or CD. Data were fit to a two-state model from dimer to monomer (\( N_2 \approx 2U \)) as described in Ref. 33, using the program Igor Pro (WaveMetrics Inc.).

For wtDCoH2 and DCoH2 S51T, unfolding kinetics were measured at GdnHCl concentrations significantly above the \( C_{u} \), so refolding rates were considered negligible (34). Kinetic unfolding data were fit to exponential unfolding curves.

DCoH2 S51Q unfolds rapidly, within minutes. Data were converted to fraction folded, as described in Ref. 22. The rates of dimer (\( N_2 \)) unfolding and monomer (\( U \)) refolding are both significant, and the data were fit to the second order differential equation

\[ \frac{dU}{dt} = -k_2U^2 - (k_1/2)U + k_3P/2 \]  

(Eq. 2)

as described in Ref. 34. In this equation, \( P \) is the total monomer concentration, \( k_1 \) is the second order rate constant for monomer folding, and \( k_3 \) is the first order rate constant for dimer unfolding. Unfolding data were fit to the equation: \( F = F_0 + F_1 (1 - U/[P_0]) \), and folding data were fit to the equation: \( F = F_0 + F_1 (U/P_0) \), with \( U \) the concentration of the unfolded protein; \( P_0 \) is 5 mM; \( F_0 \) is the CD signal of the completely folded or unfolded protein; and \( F_1 \) is the change in signal between the folded and unfolded state at each GdnHCl concentration. The values for \( F_1 \) were derived from the equilibrium fit, as described in Ref. 34. In addition, the ratio of \( k_f/k_u \) was constrained to equal the equilibrium constant at each GdnHCl concentration. Data were fit with Igor Pro (WaveMetrics Inc.) and plotted with Excel (Microsoft).

Size Exclusion Chromatography—The molecular weight of DCoH2 S51T was measured with a Superdex 75 10/300 GL gel filtration column (GE Healthcare) with PBS plus 300 mM NaCl as a mobile phase. The molecular weight was compared with standards from the low molecular weight gel filtration calibration kits (GE Healthcare). The molecular weight of DCoH S51Q was compared with wtDCoH2 in PBS with a HiPrep 16/60 Sephacryl S-200 column (GE Healthcare Life Sciences). The molecular weight was compared with Bio-Rad gel filtration standards.

**RESULTS**

**DCoH2 S51T Demonstrates Unfolding Hysteresis, Like wtDCoH1**—In wtDCoH1, Thr-51 is located at the center of the tetramer interface. In DCoH2 of humans and mice, residue 51 is a serine (1). Previously, we showed that mutating Thr-51 to Ser in DCoH1 eliminated the unfolding hysteresis (22). To confirm the contribution of Thr-51 to inducing unfolding hysteresis, we
mutated Ser-51 in DCoH2 to Thr as found in DCoH1. Although wtDCoH2 unfolds reversibly, the DCoH2 S51T mutation was sufficient to promote unfolding hysteresis in DCoH2, like wtDCoH1 (Fig. 1A). Unfolding was not concentration-dependent; refolding was concentration-dependent, as expected for an oligomer. When compared with wtDCoH2, the S51T mutation dramatically increased the unfolding transition from less than 2 M GdnHCl to about 5.5 M GdnHCl (Fig. 1B). Refolding of DCoH2 S51T remained near 2 M GdnHCl, similar to the equilibrium folding of wtDCoH2. Unfolding and refolding appear as single transitions, as measured by intrinsic Trp fluorescence. Because residue 51 is located primarily in the tetramer interface, between DCoH2 dimers, we propose that Thr-51 stabilizes the DCoH1 and DCoH2 homotramers.

**Thr-51 Displaces the Central Water Molecule**—To compare the DCoH2 S51T mutant to the wtDCoH1 structure, we crystallized DCoH2 S51T (Table 1). DCoH2 S51T crystallized in the same space group as wtDCoH2 with a dimer in the asymmetric unit, forming a tetramer with a symmetry-related dimer (1). The structure is identical to wild-type DCoH2 except near the Ser-51 mutations. The four Thr-51 residues of the homotetramer adopt the same conformation as in wtDCoH1 (Fig. 2) (20). A well ordered water molecule was apparent in the electron density of the Ser-51 structures (wtDCoH2 and DCoH1 T51S), forming hydrogen bonds with the four Ser side chains in the tetramer interface (22). This water was absent in the wtDCoH1 structure (Thr-51) (20). We proposed that this water molecule contributes to the reduced stability of the DCoH2 homotramer. In the DCoH2 S51T structure, the Thr-51 hydroxyl forms a hydrogen bond with the backbone carbonyl of Phe-47 (Fig. 2). There is a peak of density for the water molecule that is within hydrogen-bonding distance (3.0 Å) of the Thr.

**FIGURE 1.** DCoH2 S51T unfolding. A, unfolding of DCoH2 S51T exhibits hysteresis, like wild-type DCoH1 (with Thr-51) (22). Unfolding and refolding appear as single transitions, as measured by internal Trp fluorescence. Refolding (orange symbols) is concentration-dependent, as expected for an oligomer. Unfolding is not concentration-dependent, but requires over 5 M GdnHCl. Samples were equilibrated for about 20 h. DCoH2 S51T concentrations: filled diamond, 1.6 μM; open square, 4.8 μM; filled triangle, 16 μM). Error bars were derived from the standard deviation of three measurements. B, comparison of folding among DCoH2 mutants. Refolding of DCoH2 S51T (orange filled diamonds, dotted lines, 4.8 μM) is similar to the equilibrium folding curve of the wtDCoH2 (Ser-51) homotramer (black X, 4.2 μM), previously measured by Trp fluorescence (22). The black line shows the fit from a folded dimer to unfolded monomer model. The DCoH2 S51Q dimer (purple filled triangles and line, DCoH2 S51Q concentration 5 μM) measured by CD shows similar stability to wtDCoH2 (Fig. 6). Unfolding of DCoH2 S51T is shown as blue filled diamonds, connected by dotted lines.
The B factor of this H2O (water 230) refined to 31.4 Å², significantly greater than the B factor of the Thr-51 OG1 hydroxyls it would be hydrogen-bonded to (≈13 Å²). When the B factor of water 230 was fixed at 13 Å², the occupancy decreased to 0.1 before the negative electron density disappeared in the \( F_o - F_c \) map. This water is located on a special position, between the dimers of the homotetramer. Therefore if a water molecule is present at the tetramer interface of DCoH2 S51T, the occupancy is low.

**Unfolding Kinetics of wtDCoH2 and DCoH2 S51T**—To determine whether the unfolding hysteresis of DCoH2 S51T could be explained by slow unfolding of the homotetramer, we compared the unfolding rates of wtDCoH2 and the DCoH2 S51T mutant. Kinetic unfolding data were collected for wtDCoH2 at >1.4 M GdnHCl (Fig. 3, A and C). At GdnHCl concentrations significantly greater than the \( C_m \), unfolding was much faster than folding, and the data were fit to a first order equation or to exponential unfolding with time (Fig. 3B shows the residuals for the fits to the fluorescence data) (34). The unfolding rate constants measured by circular dichroism were consistent with the fluorescence measurements at 2, 3, and 4 M GdnHCl (Fig. 3D).

The log of the observed rate constant was linear above 0.5 M GdnHCl. At lower GdnHCl concentrations, refolding was significant. Extrapolation of the unfolding rate constant to 0 M GdnHCl indicated that the wtDCoH2 homotetramer unfolds in aqueous solution with \( k_{\text{unfolding}} = 2 \times 10^{-5} \text{ s}^{-1} \), or with an unfolding half-time \( \tau_{\text{unfolding}} = 9.4 \text{ h} \).

The unfolding rate of the DCoH2 S51T mutant was significantly slower than for wtDCoH2 (Fig. 4). The Thr-51 mutant required high GdnHCl concentrations to unfold; fluorescence data were measured at greater than or equal to 4 M GdnHCl (Fig. 1). The data were fit to a double exponential unfolding model (Fig. 4, A and B). Fig. 4C shows the residuals for the fits. The plot of \( \ln(k_{\text{unfolding}}) \) versus GdnHCl indicated that the fast rate constant was independent of GdnHCl concentration and was \( \sim 10^{-4} \text{ s}^{-1} \) (Fig. 4E). The slow rate was linear versus GdnHCl at 5 M GdnHCl and above; refolding was significant at less than 5 M GdnHCl. The observed unfolding rate constant measured by CD at 5, 5.5, and 6 M GdnHCl was the same as the slower rate constant measured by fluorescence, suggesting that the slow rate represents global unfolding (Fig. 4, D and E). We interpret the fast rate constant measured by fluorescence as a local unfolding effect in the environment of one of the Trp residues, possibly Trp-7, which is exposed to solvent and is not present in DCoH1 (Fig. 2A) (22). Extrapolating the slower unfolding rate constant to 0 M GdnHCl resulted in \( k_{\text{unfolding}} = 1.4 \times 10^{-14} \text{ s}^{-1} \), 9 orders of magnitude slower than the unfold-
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Unfolding of a DCoH2 Dimer, DCoH2 S51Q—To measure unfolding of the DCoH2 dimer, we mutated Ser-51 in the tetramer interface to Gln. In silico the Gln residues clash across the tetramer interface. The Gln side chain is also hydrophilic, reducing stability of the tetramer. Formation of the DCoH2 S51Q dimer was confirmed by size exclusion chromatography (Fig. 5).

Equilibrium unfolding by GdnHCl was measured by intrinsic Trp fluorescence and CD at 5 μM monomer concentration (Fig. 6). Superposition of the folding and unfolding curves confirmed that the samples had reached equilibrium within 12 h of an overnight incubation (Fig. 6A). The fluorescence and CD unfolding measurements agreed, with a $C_m$ of ~1.5 M GdnHCl (Fig. 6B). We fit the equilibrium unfolding curve to a two-state model: from folded dimer to unfolded monomers, as described in Ref. 22. The parameters for the best fit are listed in the figure legend. The Δ$G_\text{H2O}$ and $m$-values were: Δ$G_\text{H2O} = 66.0$ kJ/mol, $m = 21.3$ kJ/mol × M. The dissociation constant for DCoH S51Q in aqueous solution = exp(−Δ$G_\text{H2O}/RT$) = 2.65 × 10^{-12} M.

Kinetic folding and unfolding data were measured for DCoH2 S51Q by CD (Fig. 7). Folding times were much faster than for wtDCoH2 or DCoH2 S51T, on the order of a minute. Data were collected manually for the slowest rates, close to the $C_m$ value, for which folding and unfolding rates are significant. There-

FIGURE 3. Kinetics of unfolding of wtDCoH2 (Ser-51). A, unfolding kinetics measured by intrinsic Trp fluorescence at 1.4, 1.6, 1.8, 2.0, 3.0, and 4.0 M GdnHCl at 1.6 μM DCoH2 monomer measured for 24 h. The curves show the fits to single exponential equations. B, residuals for the single exponential fits to the fluorescence unfolding data. The symbols are the same as in A. The residuals have been translated up in units of 1 nm so they do not overlap. C, unfolding kinetics data measured by CD at 2.0, 3.0, and 4.0 M GdnHCl, fit to a single exponential equation. mdeg, millidegrees. D, lnk versus GdnHCl. The dotted line is drawn through the points for which refolding is negligible. The line extrapolates to lnk = −10.8 at 0 M GdnHCl, or $k = 2 \times 10^{-3}$ s. Symbols: X, fluorescence rate constants; △, CD rate constants. The fluorescence and CD rate constants overlap. E, table showing the best fit of the data to the single exponential equation: $y = y_0 + A_1 \exp(-k_1 t)$, chsqa, goodness of fit defined as $\Sigma (y - y)^2$.

| GdnHCl (M) | $y_0$ | $A_1$ | chsqa |
|------------|-------|-------|-------|
| 1.4        | 342.67 ± 0.05 | -0.60 ± 0.2 | 1.4 ± 1.0|
| 1.6        | 344.65 ± 0.06 | -2.60 ± 0.2 | 1.3 ± 0.2|
| 1.8        | 350.70 ± 0.2  | -3.60 ± 0.2 | 1.8 |
| 2.0        | 352.32 ± 0.09 | -10.92 ± 0.08 | 2.5 |
| 3.0        | 352.43 ± 0.06 | -11.50 ± 0.07 | 0.3 |
| 4.0        | 352.50 ± 0.06 | -12.15 ± 0.09 | 0.4 |
| CD data    | -3.61 ± 0.02 | -13.85 ± 0.02 | 23.6 |
| 3.0        | -2.09 ± 0.02 | -14.61 ± 0.02 | 48 |
| 4.0        | -0.22 ± 0.01 | -17.67 ± 0.02 | 16 |

* fluorescence data 31 points
* CD data 1000 points
fore the data were fit to a reversible model between folded dimer and unfolded monomer, as described in Ref. 34. The kinetic curves were fit globally to find the best values for $k_{\text{unfolding}}$ and $k_{\text{folding}}$. The fits to 8 kinetic curves (duplicates of unfolding curves at 1.67, 2.07, and 3.07 M GdnHCl and refolding curves at 1.67 and 2.07 M GdnHCl, 972 total data points) were fit with an overall $r^2$-squared value of 4.1 (Fig. 7). The rate of monomer folding to dimer, extrapolated to aqueous solution, is fast: $5 \times 10^{-7}$ M$^{-1}$ s$^{-1}$, 2 orders of magnitude lower than the diffusion limit of $10^9$ M$^{-1}$ s$^{-1}$. The extrapolated dimer unfolding rate is $1.4 \times 10^{-4}$ s$^{-1}$, 10 times faster than unfolding of the wtDCoH2 (Ser-51) homotetramer. Although the fit to the data is good, it is important to note that the kinetic parameters for the dimer are only approximate because they were derived from a small range of data close to the unfolding midpoint.

DISCUSSION

The mammalian DCoHs are bifunctional proteins that function both as metabolic enzymes (recycling tetrahydrobiopterin) and as transcriptional coactivators (interacting with the HNF-1
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transcription factors, HNF-1α and HNF-1β) (10). The two functions of DCoH are associated with a change in oligomeric state: from a homotetramer in the cytoplasm to a dimer in the nucleus interacting with a dimer of HNF-1 (20, 21). To determine how DCoH partitions between these two apparently unrelated activities, we are characterizing the stability of the DCoH homotetramer. Previous equilibrium unfolding measurements comparing the two DCoH paralogs, DCoH1 and DCoH2, demonstrated that DCoH2 unfolds reversibly, whereas DCoH1 exhibits unfolding hysteresis (22). Mutating a single residue in DCoH1 (Thr-51) to that of DCoH2 (Ser-51) was sufficient to overcome the unfolding hysteresis of wtDCoH1. In the current study, we provide evidence that wtDCoH1 (Thr-51) is kinetically stable, explaining the inability of the DCoH1 homotetramer to dissociate and interact with HNF-1 in vitro (21, 27, 28).

Kinetic Stability of DCoH Thr-51—To confirm the role of Thr in stabilizing the DCoH homotetramer, we mutated Ser-51 to Thr in DCoH2. Like wtDCoH1, Thr-51 was sufficient to introduce unfolding hysteresis to DCoH2 (Fig. 1). The four Thr-51 residues in the tetramer interface stabilize the DCoH2 homotetramer (Fig. 2). According to kinetic unfolding measurements, the Ser-51 Thr mutation reduces the unfolding rate of DCoH2 by 9 orders of magnitude: from $2 \times 10^{-9}$ s$^{-1}$ ($\tau_{1/2} = 9$ h) to $1.4 \times 10^{-14}$ s$^{-1}$ ($\tau_{1/2} = 1.6$ million years) (Figs. 3 and 4). For comparison, α-lytic protease is kinetically trapped with a half-life of 1.2 years (35, 36). Kinetic stability of DCoH Thr-51 explains the unfolding hysteresis.

All equilibrium unfolding transitions of DCoH1 and DCoH2 appear as single transitions as measured by intrinsic Trp fluorescence and CD. This is likely because the dimer and homotetramer of DCoH have the same fluorescence and CD signals, masking the tetramer to dimer transition. The two Trp residues of DCoH1, as well as the three Trp residues of DCoH2, are located within the dimer, away from the tetramer interface (Fig. 2) (22). The far UV CD spectra of the dimer and tetramer are expected to be similar, with little change in secondary structure.
The DCoH2 S51Q mutation was designed to compare the stability of the DCoH2 dimer with the homotetramer. Although it is possible that Gln-51 destabilizes the DCoH2 dimer relative to wtDCoH2, the DCoH2 S51Q mutant forms a stable dimer as measured by size exclusion chromatography (Fig. 5). The stability of DCoH2 S51Q is $\Delta G_{\text{refolding}} = 66 \text{ kJ/mol (K}_\text{d} = 2.6 \times 10^{-12} \text{ M}$, similar to wtDCoH2 fitted to a dimer to unfolded monomer model ($\Delta G_{\text{dimer}} = 73 \text{ kJ/mol}, K_\text{d} = 1.6 \times 10^{-13} \text{ M}$), and the refolding transition of DCoH2 S51T (Fig. 1) (22). The DCoH2 S51Q dimer unfolds much faster than the homotetramer: $k_{\text{unfolding}} = 1.4 \times 10^{-1} \text{ s}^{-1}$ ($\tau_\text{d} = 1.4$ h). Therefore the current data are consistent with a three-state folding model from unfolded monomers to dimers to folded homotetramer. wtDCoH2 and DCoH2 S51T homotetramers are kinetically more stable than the DCoH2 dimer. Once the homotetramer unfolds to dimers, the dimers unfold rapidly.

**Model for Kinetic Stability, a Kinetic Hot Spot—Ser to Thr is a conservative mutation, only adding a methyl group to each Ser-51 of the homotetramer. Given the dramatic effect of this mutation on the unfolding kinetics of DCoH, we propose that residue 51 is a kinetic hot spot, affecting the off-rate of the homotetramer more than the equilibrium constant (37).

According to the solvation/desolvation model for kinetic stability, the barrier to unfolding results from intramolecular interactions that are broken during unfolding, but not yet compensated by solvation (38). For the DCoH interface, these interactions are primarily hydrophobic and van der Waals contacts with a few hydrogen bonds on the periphery of the interface (1). A total of 2,400 Å$^2$ of the surface area is buried from both DCoH dimers to form the homotetramer (22). The relatively flat and rigid tetramer interface of DCoH may contribute to the large solvation barrier. In this model, the presence of the central water molecule bound to Ser-51 facilitates solvation during unfolding, lowering the unfolding barrier. Similar to DCoH, kinetic stability of $\alpha$-lytic protease is due to rigidity in the structure and cooperative unfolding (39, 40). Some thermophilic proteins unfold much more slowly than their mesophilic orthologs (41). It would be worth probing these proteins for similar kinetic hot spots.

**Functional Consequences of Kinetic Stability—Kinetic stability of the DCoH1 homotetramer allows DCoH to adopt two distinct functions. Because the enzymatically active cytoplasmic pool of PCD1 (DCoH1) homotetramers will not mix with the transcriptionally active nuclear pool of DCoH1-HNF-1 complexes, the two pools can be maintained at distinct concentrations. In hepatocytes, DCoH1 is found at high concentration in the cytoplasm ($\sim 6 \mu\text{M}$) and at a much lower concentration in the nucleus (11, 17). The PCD1 homotetramer must be maintained at high concentration in the cytoplasm to bind its 4α-hydroxytetrahydrobiopterin substrate and prevent spontaneous dehydration to 7-tetrahydrobiopterin (11, 42). Accumulation of 7-tetrahydrobiopterin causes a mild form of hyperphenylalaninemia (43, 44).

According to the EMBL-EBI Expression Atlas, DCoH2 (PCBD2) is typically expressed at 5–20-fold lower levels than DCoH1 (PCBD1) in many organs (compare ENSG00000132570 and ENSG00000166228) (45). The DCoH2 homotetramer may therefore be available to assure low levels of DCoH activity for example interacting with HNF-1 as shown in DCoH1 null mutant mice (25).

Disease-associated mutations have been identified in DCoH1, not DCoH2. Mutations in DCoH1 have long been known that affect its enzymatic function, causing mild hyperphenylalaninemia (25, 42, 47). Recently, homozygous mutations in DCoH1 were found that disrupt its coactivator function. In the kidney, mutations in DCoH1/PCD1 decrease transcriptional activity of HNF-1 and 1β, resulting in reduced magnesium absorption and diabetes (48, 49). DCoH1 interacts with the N-terminal dimerization domain of HNF-1 (21). Interestingly, mutations in HNF-1β far from the dimerization domain alter the amount of DCoH1 in the nucleus. Ferre et al. (48) explain these mutations as destabilizing the DCoH-HNF-1β complex in the nucleus. The kinetic stability of the DCoH1 homotetramer suggests that the amount of complex in the nucleus is determined during synthesis of the complex in the cytoplasm, upon folding. We therefore suggest that the HNF-1β mutations affect formation of the complex with DCoH1, for example affecting the rate of folding of HNF-1β.

In the pancreas, heterozygous mutations in DCoH1 that cause mild neonatal hyperphenylalaninemia also increase the risk of monogenic diabetes (50). Mutations in HNF-1α cause maturity-onset diabetes of the young type 3 (MODY3) (51). Reduced levels of DCoH1 may affect formation of the DCoH1-HNF-1 complex and HNF-1α activity, reducing beta cell mass. Future studies will address how mutations in DCoH1 and in HNF-1 affect DCoH1-HNF-1 complex formation in vivo. Conclusion—We show that a Ser-51 to Thr mutation in the tetramer interface of DCoH2 results in unfolding hysteresis due to slow unfolding of the homotetramer. Residue 51 is a kinetic hot spot, an interface residue that alters the dissociation kinetics dramatically when mutated. One characteristic of kinetic hot spots may be the exclusion of water from the interface. Kinetic stability allows DCoH to adopt two independent functions. We propose that mutations in DCoH or HNF-1 that cause defects in the kidney or pancreas can result from abnormal partitioning of DCoH1 between its enzymatic and coactivator functions.

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