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Can the DCoHα isozyme compensate in patients with 4a-hydroxy-tetrahydrobiopterin dehydratase/DCoH deficiency?

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Abstract

4a-Hydroxy-tetrahydrobiopterin dehydratase/DCoH is a bifunctional protein. In the cytoplasm it is an enzyme required for the regeneration of tetrahydrobiopterin, an essential cofactor for phenylalanine hydroxylase. In the nucleus it functions as a transcriptional coactivator by forming a 2:2 heterotetramer with the hepatic nuclear factor HNF1α (HNF1). Patients with a deficiency of dehydratase activity have elevated levels of phenylalanine, and accumulate 7-pterins due to degradation of its substrate 4a-hydroxy-tetrahydrobiopterin. Curiously, the hyperphenylalaninemia is transient, and no defects in the transcriptional coactivator function have been reported. Recently, a human isozyme, dehydratase/DCoHα, has been detected which shares 60% identity with dehydratase/DCoH. This investigation was undertaken to ascertain if dehydratase/DCoHα has the pre-requisite properties to compensate in individuals lacking an active form of DCoH. DCoHα demonstrated the ability to quantitatively alter HNF1-dependent DNA-binding in vitro whereas DCoH was ineffective in vitro. This characteristic, due to the presence of dimeric DCoHα, demonstrates that DCoHα does not require any additional mammalian regulation process to alter DNA binding and therefore, may be more effective than DCoH at low concentrations. The dehydratase activity of each isoform was measured by a direct spectrophotometric assay. K_m and V_{max} for DCoHα were both 2–3 times higher than for DCoH, thus leaving the catalytic efficiency (V_{max}/K_m) the same for both enzymes. In conclusion, the properties of dehydratase/DCoHα are consistent with the hypothesis that the activity of this isozyme could account for the relatively mild symptoms reported for patients with a defect in dehydratase/DCoH.

Keywords: 4a-Hydroxy-tetrahydrobiopterin dehydratase; Hyperphenylalaninemia; DCoH; DCoHα; HNF1α; Pterin carbinolamine dehydratase; PCD; Bifunctional protein; Tetrahydrobiopterin; 7-Substituted pterins

Introduction

4a-Hydroxy-tetrahydrobiopterin dehydratase/DCoH[2,3] [EC 4.2.1.96] is a bifunctional protein with two totally different roles dependent on its state of oligomerization (Fig. 1). As a stable homotetramer, it is a cytoplasmic enzyme which regenerates the tetrahydrobiopterin cofactor of phenylalanine hydroxylase [1]. The second function of this protein is in the nucleus where it activates transcription as the dimerization cofactor (DCoH) of hepatic nuclear

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[2] *Abbreviations used:* ApoB, apolipoprotein B; DCoH, dimerization cofactor of HNF1; DCoHα, Accession No. NM 032151.3 (also referred to as DCoH2 and DCoHm); DHPR, dihydropteridine reductase; EMSA, electromobility shift assay; HNF1, hepatocyte nuclear factor 1α; HNFt, truncated HNF1 consisting of the first N-terminal 281 amino acids; HRE, HNF1 response element; HPA, hyperphenylalaninemia; IPGT, isopropyl-B-D-1-thiogalactopyranoside; NC1 and NC2, negative control duplexes 1 and 2; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride, tetrahydrobiopterin, 6(*R*)-*L*-*erythro*-dihydroxy-propyltetrahydropterin; WT, wild-type.
[3] 4a-Hydroxy-tetrahydrobiopterin dehydratase (EC 4.2.1.96) is also known as 4a-hydroxy-tetrahydropterin dehydratase, pterin 4a-carbinolamine dehydratase, and DCoH.

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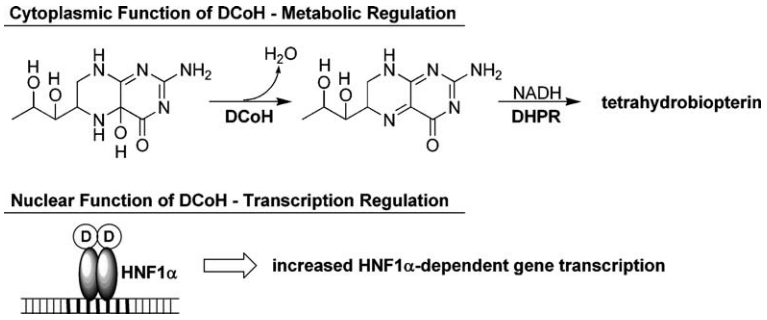

Fig. 1. Dehydratase/DCoH is a bifunctional protein. In the cytoplasm it acts as a dehydratase in concert with dihydropteridine reductase (DHPR) to regenerate tetrahydrobiopterin, an essential cofactor for the aromatic amino acid hydroxylases. In the nucleus as DCoH (D) (dimerization cofactor of HNF1) it functions as a co-activator of HNF1-dependent transcription.

transcription factor 1α (HNF1) [2,3]. To bind to DNA, HNF1 must be in a dimeric form [4]. The transcriptional activation by DCoH is entirely through protein–protein interactions, since DCoH itself does not bind to DNA [5]. The active complex is a heterotetramer composed of 2 subunits of HNF1 and 2 subunits of DCoH bound to DNA [2]. HNF1 regulates the transcription of a large number of genes, not only in the liver where it was first identified, but also in the kidney, pancreas, and other tissues [6,7]. Loss of the HNF1 protein in HNF1 null mice has been associated with renal carcinogenicity [8] and mutations in the HNF1 gene are associated with maturity onset diabetes of the young [9]. Thus, proper maintenance of HNF1-specific gene transcription is critical.

Patients with mutations in the dehydratase/DCoH gene (PCBD1; Accession No. NM 000281) have transient hyperphenylalaninemia [10,11], and excrete elevated levels of 7-pterins [12]. However, these individuals develop and grow normally with no symptoms of deficiency in the transcriptional co-activator function of DCoH. Recently, an isozyme of DCoH, which was first identified in chicken [13], has also been found in humans [14,15] (Accession No. NM 032151.3). The amino acid sequence of the human isozyme, here denoted as DCoHα, is 60% identical to DCoH. The high level of dehydratase in normal liver tissue (4–6 μM) is required to handle a phenylalanine load [16,17]. In normal individuals, this activity is primarily, if not totally, provided by dehydratase/DCoH. Therefore, the transient nature of this metabolic disorder has remained unclear.

This investigation was undertaken to ascertain whether the properties of DCoHα could account for the symptoms of patients with defective dehydratase/DCoH. Here, we have characterized the ability of DCoH and DCoHα to alter HNF1-dependent DNA binding, and have compared the enzymatic activity of both isoforms. Although both DCoH [2] and DCoHα [14] are capable of activating HNF1-dependent transcription in transient transfection studies, if DCoHα is more efficient than DCoH, it could explain the lack of disruption of transcriptional activity in dehydratase/DCoH defective patients even at very low DCoHα concentrations. Furthermore, if DCoHα has sufficient catalytic activity, the transient nature of the hyperphenylalaninemia, observed in newborns with mutations in the dehydratase/DCoH gene, could be due to the time required to induce dehydratase/DCoHα to a level adequate to maintain a supply of tetrahydrobiopterin for phenylalanine hydroxylation.

Materials and methods

General methods

Dehydratase activity assays were performed at 10 °C in 25 mM BES-Na, pH 7.4, with 4a-hydroxy-6(*S*)-methyltetrahydropterin as the substrate as previously reported [18,19]. Under these conditions the non-enzymatic rate of dehydration is 0.006 s^{-1}. DCoH, DCoH/HNFt, and DCoHα/HNFt protein concentrations were determined either by the method of Lowry [20] or by spectral characterization using molar extinction coefficients as described previously [18]. The concentrations of HNFt and DCoHα were determined using the DC Protein Assay (Bio-Rad). All protein purification steps were performed at 4 °C. Unless otherwise specified, buffer exchange was done by centrifugation in CentriconPlus-20 (30,000 MWCO or 10,000 MWCO) filter units. Mass spectrometric analyses were performed at the University of South Alabama Proteomics and Mass Spectrometry Research Facility.

Oligonucleotide and duplex DNAs

The following oligonucleotides were synthesized by either Operon, or Integrated DNA Technologies, and used in PCR amplifications: DCoHαfor, 5′-GCA GAT ATC ATA TGT CAT CAG GTA CTC ACA GGT TG; DCoHαrev, 5′-CGG GAT CCT ACA CAG AAG CAG CTG CTT TTT C; Hfor, 5′-CGG AAT TCA TAT GGT TTC TAA ACT GAG CC; and Hrev, 5′-CGG GAT CCT ACA GCT TGT GCC GGA A. The following oligonucleotides, with or without 5′-biotin labeling, were synthesized and HPLC purified by Integrated DNA Technologies: ApoBfor, 5′-CTG TCC TGT TTA TCA GTG ACT AGT CAT; ApoBrev, 5′-ATG ACT AGT CAC TGA TAA ACA GGA CAG; NC1for, 5′-CAC TGC CCA GTC AAG TGT TCT TGA; NC1rev, 5′-TCA AGA ACA CTT GAC TGG GCA GTG; NC2for, 5′-ATG AAT AAT CCC TTC GAC GAG AGA AAG; and NC2rev, 5′-CTT TCT CTC GTC GAA GGG ATT ATT CAT. The HNF1-binding site (HRE) is underlined. The concentration of each oligonucleotide stock solution was determined by UV spectroscopy and the calculated absorption coefficients of each oligonucleotide. Hybridization of complimentary strands was accomplished by heating equal concentrations of the oligonucleotides to 94 °C for 5 min followed by cooling to room temperature.

Purification of recombinant human wt dehydratase/DCoH

The expression vector pGEMEX-2-Nde/DCoH and the procedure for overexpressing DCoH in *Escherichia coli* BL21(DE3)pLysS cells has been

described previously [18]. The published purification procedure for DCoH [18] was modified by replacing the chromatofocusing step with anion exchange chromatography at 4 °C as follows. Soluble 100,000*g* extracts (~50 mg protein aliquots) in 5 mM Tris–Cl, pH 8.0, were applied to a 5 mL HiTrapQ column (Pharmacia). The column was washed with 20 mM bis–Tris–Cl, pH 7.4, and DCoH was eluted with a pH gradient from pH 7.4–6.0. Samples were stored as described previously [18].

Overexpression and purification of recombinant human DCoHα

The DNA encoding DCoHα (also referred to as DCoHm [14] and DCoH2 [15]) was amplified from the pGTEX-4T-1-DCoHm plasmid (a kind gift from E. Friedman) by PCR using *Pfu* polymerase and primers corresponding to the 5′ end with additional *Eco*RV/*Nde*I sites and the 3′ end with an additional *Bam*HI site (see DCoHαfor and DCoHαrev in the *Oligonucleotide* section). The PCR product was digested with *Eco*RV/*Bam*HI, ligated into pBlueScriptIIKS+, amplified and sequenced. The DNA was then digested with *Nde*I/*Bam*HI and ligated into pGEMEX-2-*Nde* that was previously cut with *Nde*I/*Bam*HI. The resulting vector, referred to as pGEMEX-2-*Nde*/DCoHα was used to transform *E. coli* BL21(DE3)pLysS cells. Cells were collected and extracted as for DCoH. DCoHα was purified using a p-11 phosphocellulose (Whatman) column (Supplementary data) and stored in 50 mM Hepes, pH 7.8, 100 mM KCl, and 10% (v/v) glycerol at −80 °C. Preparations of DCoHα were between 80 and 95% pure as judged by SDS–PAGE. The identity of DCoHα was confirmed by mass spectrometric analysis.

Overexpression and purification of recombinant human HNFt

A truncated version of HNF1, comprising the N-terminal 281 amino acids and referred to as HNFt has been described previously [5]. The DNA encoding HNFt was amplified from the pHA plasmid (a mammalian expression vector for human HNF1 and a kind gift from M. Pontoglio) by PCR using *Pfu* polymerase and primers corresponding to the 5′ end with additional *Eco*RI/*Nde*I sites and the 3′ end with an additional *Bam*HI site (see Hfor and Hrev in the *Oligonucleotide* section). The PCR product was digested with *Eco*RI/*Bam*HI, ligated into pBlueScriptIIKS+ (Stratagene), amplified and sequenced. The DNA was digested with *Nde*I/*Bam*HI and ligated into pET30b (Novagen) that was previously cut with *Nde*I/*Bam*HI to remove DNA encoding a fusion protein. The resulting vector, referred to as pETK/HNF was used to transform *E. coli* BL21(DE3)pLysS cells. A soluble protein supernatant from IPTG-induced cells was prepared by resuspending the cell pellet in 20 mM Tris–HCl, pH 8.8, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 0.1 mM benzamidine, sonication and centrifugation. HNFt was purified using ammonium sulfate precipitation, and heparin Sepharose and Mono S Sepharose columns (Supplementary data). Fractions containing HNFt were concentrated and stored in 50% glycerol at −20 °C. Preparations of HNFt were between 80 and 90% pure as judged by SDS–PAGE. The active protein concentration was determined by titrating HNFt with DNA, followed by EMSA analysis as discussed below.

Overexpression and purification of recombinant DCoH/HNFt and DCoHα/HNFt complexes

The DCoH/HNFt complex was formed by co-expression of the pGEMEX-2-*Nde*/DCoH and pETK/HNF plasmids in *E. coli* BL21(DE3) pLysS cells. DCoH/HNFt complex was purified to ~90% by p-11 phosphocellulose and phenyl-Sepharose chromatography as previously described [5]. The DCoHα/HNFt complex was formed by co-expression of the pGEMEX-2-*Nde*/DCoHα and pETK/HNF plasmids in *E. coli* BL21(DE3)pLysS cells. Cells were extracted by sonication and the DCoHα/HNFt complex purified by a modification of the published procedure for the DCoH/HNFt complex (Supplementary data). Preparations of DCoHα/HNFt were between 85 and 90% pure as judged by SDS–PAGE. Concentrated proteins were frozen in aliquots in liquid nitrogen and stored at −80 °C.

HPLC

Protein separations were performed at room temperature on a Phenomonex BioSep SEC-S3000 (300 × 7.8 mm) column using 50 mM Tris–acetate, 150 mM sodium acetate, pH 7.3, as a mobile phase at a flow rate of 0.6 mL/min. Column eluants were monitored by fluorescence detection (280 nm excitation/350 nm emission) with a JASCO FP1520 or Shimadzu RF-10AXL fluorometer.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assay (EMSA) was utilized to assess the binding of various protein species to three different duplex DNAs: (1) duplex DNA containing an HRE from the apolipoprotein B promoter (ApoB) [21], (2) a negative control duplex (NC1) previously shown not to bind to HNFt [22], and (3) a negative control duplex (NC2) that is equivalent in length and base composition to ApoB but does not contain the HRE. A standard-binding reaction was performed at room temperature for 25 min. Time course studies demonstrated that equilibrium was reached in less than 15 min. Unless noted otherwise, the reaction mixture (10 μL) consisted of 10 nM biotin-labeled duplex DNA, 20 mM Tris–HCl, pH 8, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 1% (v/v) glycerol ± competitor DNA (as per Fig. 3) and was initiated with protein as stated in the figures. Proteins were diluted just before use in 50 mM Hepes, pH 7.6, 150 mM KCl, 1 mM EDTA, and 1 mM DTT. The protein–DNA complexes were separated on a pre-electrophoresed 6% DNA retardation gel (Invitrogen) run in 0.5× TBE buffer (0.045 M Tris–borate/0.001 M EDTA) at 100 V for 45–60 min. DNA was visualized using the LightShift Chemiluminescent EMSA Kit (Pierce) according to manufacturer instructions. Bound and free DNA were visualized using either autoradiography or a Kodak ImageStation 2000R or a Fuji LAS-1000 and quantitated using ImageGauge software (Fuji). Titration reactions were performed with 125 nM HNFt or DCoH(α)/HNFt and a varying amount of ApoB duplex. HNFt concentrations were corrected for that portion of protein determined to be active by titration analysis performed on the same day that each binding experiment was performed. BSA at a final concentration of 500 nM was added to both titration and binding curve experiments to stabilize HNFt. In experiments involving mixing of DCoH(α) with HNFt, DCoH(α) was added at 125 nM (final concentration of tetramer) just prior to initiation with HNFt. Components of DCoHα storage buffer (KCl and glycerol) were shown not to affect the binding isotherm when used in amounts normally present with DCoHα.

Results

Oligomeric state of DCoH and DCoHα

It has previously been shown [18] that DCoH is a stable tetramer, even at concentrations down to 1 nM, yet the active complex with HNF1 is a heterotetramer (2DCoH/2HNF). Attempts to concentrate purified DCoHα using filtration devices with 30,000 MWCO yielded much of the protein in the filtrate, suggesting that DCoHα was not a stable tetramer. This is in contrast to DCoH which is all found in the retentate under similar conditions. The oligomeric state was further investigated by size exclusion chromatography. Unlike DCoH, which elutes from the column as a single narrow peak [18] corresponding to a tetramer regardless of concentration, the elution pattern of DCoHα was highly dependent upon protein concentration (Fig. 2). At very high DCoHα concentrations, the protein eluted as a single peak with some trailing and a retention time similar to DCoH indicating mostly a tetrameric species. However,

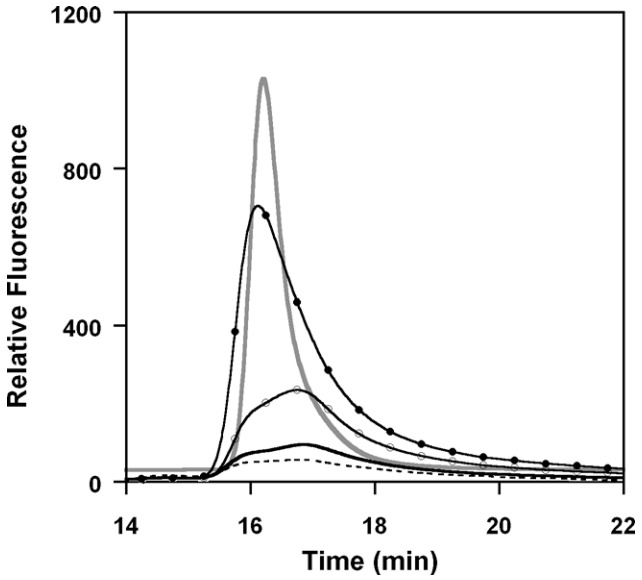

Fig. 2. Quaternary structure of DCoH and DCoHα; DCoHα is not a stable tetramer. Samples of the following DCoHα concentrations (in subunits) were prepared in HPLC mobile phase, and 10 μL of each injected: 84 μM (solid with filled circles); 30 μM (solid with open circles); 6 μM (bold solid); and 2 μM (dashed) and chromatographed on a Biosep SEC-S3000 (Phenomenex) column. The gray line is DCoH (6 μM) with the fluorometer scale expanded 10-fold.

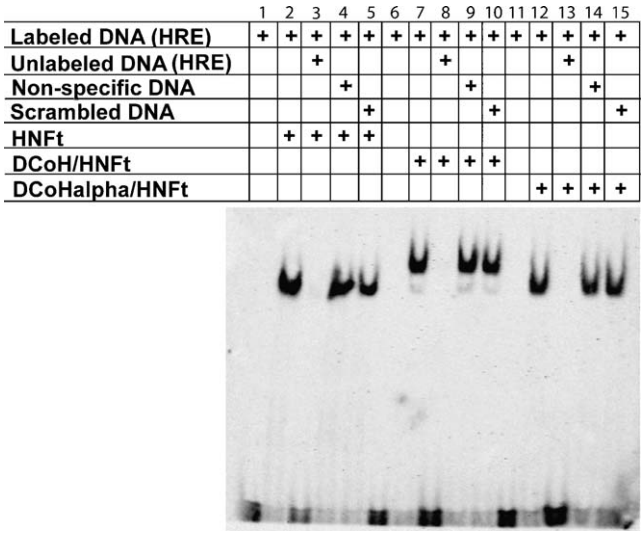

Fig. 3. Specific binding of HNFt, HNFt/DCoH, and HNFt/DCoHα to the HNF response element (HRE) in the ApoB promoter. DCoH/HNFt and DCoHα/HNFt complexes were produced by co-expression in *E. coli*. Biotin-labeled ApoB duplex was mixed with the various proteins in the presence of either unlabeled ApoB (lanes 3, 8, and 13), a non-specific DNA sequence (lanes 4, 9, and 14) or a scrambled HRE sequence (lanes 5, 10, and 15). Lanes 1, 6, and 11 did not contain HNFt. Protein–DNA complexes were separated from labeled probe electrophoretically (run time 35 min at 80 V). Only the unlabeled ApoB HRE competed with the labeled ApoB.

at lower concentrations of DCoHα, lower molecular weight species were observed in the profile, indicating dissociation into dimers and/or monomers.

Comparison of the ability of DCoH and DCoHα to affect binding of HNFt to DNA

In its nuclear function as a transcriptional coactivator, DCoH forms a 2:2 heterotetramer with HNF1. Although DCoHα is primarily dimeric at low concentrations, DCoH exists as a stable tetramer (Fig. 2). This difference results in a dramatic effect on the HNFt-binding capabilities of the two isoforms, as shown in the following experiments.

The ability of human DCoHα to alter the binding of HNF1 to DNA in vitro was determined using a truncated version of HNF1 (HNFt), and a 27 bp DNA duplex (ApoB) containing the sequence from the second-intron enhancer region of the human apolipoprotein B promoter (nucleotide positions +839 to +865) as the HRE [23]. Electrophoretic gel mobility shift assays (EMSAs) (Fig. 3) show binding of HNFt, DCoH/HNFt[4] (prepared by co-expressing the two proteins) and DCoHα/HNFt (prepared by co-expressing the two proteins) to biotin-labeled ApoB (lanes 2, 7, and 12, respectively). Unlabeled ApoB could compete for binding (lanes 3, 8, and 13). Neither a non-specific DNA sequence (lanes 4, 9, and 14) nor a scrambled HRE sequence (lanes 5, 10, and 15) competed with the labeled HRE. In the absence of HNFt, neither DCoH nor DCoHα bound to ApoB duplex (Fig. 4). These experiments demonstrate sequence-specific binding of purified HNFt, DCoH/HNFt, and DCoHα/HNFt to the HRE in the ApoB promoter.

A band corresponding to the protein–DNA complex from DCoH/HNFt prepared by co-expressing the two proteins (lane 6, Fig. 5) consistently ran higher in the gel than HNFt alone (lane 2, Fig. 5), suggesting the presence of DCoH in the HNFt–DNA complex. EMSA-Western experiments confirmed the presence of DCoH in the protein–DNA complex (data not shown). Similar results were obtained with DCoHα/HNFt prepared by co-expressing the two proteins (lane 7, Fig. 5). These results show that the bacterially co-expressed DCoH/HNFt and DCoHα/HNFt complexes bound to DNA. Furthermore, the binding in each case must be extremely tight, since the complexes remained intact during DNA-binding procedures and native gel analysis. Addition of purified DCoHα to HNFt in DNA-binding assays (lane 5, Fig. 5) resulted in a protein/DNA complex that co-migrated with in vivo-prepared DCoHα/HNFt (lane 7, Fig. 5), demonstrating an interaction between HNFt and DCoHα in vitro that was stable during the DNA-binding reaction and subsequent gel electrophoresis. In contrast, DCoH did not bind when added to HNFt and DNA in vitro (Fig. 5, lanes 2–4), suggesting that in vivo a cell mediated event is required before DCoH can form a complex with HNF1.

The potential biological significance of the binding of DCoHα to HNFt and DNA in vitro was probed using

[4] "DCoH/HNFt" is used to refer to functional heterotetrameric complex made in vivo by coexpression whereas "DCoH + HNFt" refers to a solution where purified DCoH is mixed with purified HNFt in vitro. The same terminology is used for DCoHα.

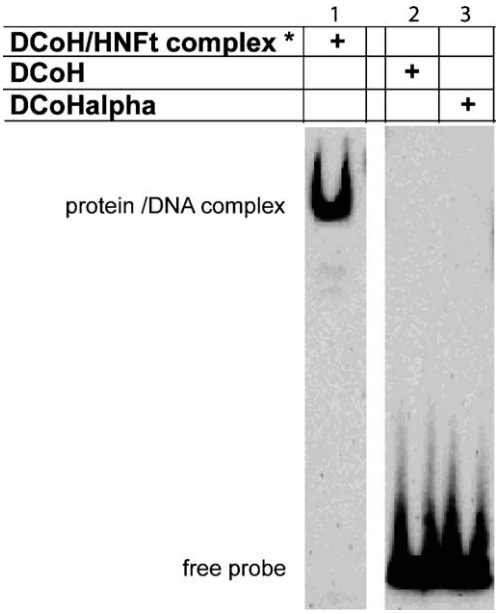

Fig. 4. DCoH and DCoHα do not bind to the HNF1 HRE in the ApoB promoter. Electromobility shift assays demonstrate that DCoH/HNFt (750 nM, lane 1), but not DCoH (500 nM, lane 2) or DCoHα (500 nM, lane 3), forms a complex with the ApoB duplex (20 nM in each reaction). *DCoH/HNFt complex was produced by co-expression in *E. coli*. DNA was visualized using Sybr Green (Molecular Probes/Invitrogen) (run time 35 min at 100 V).

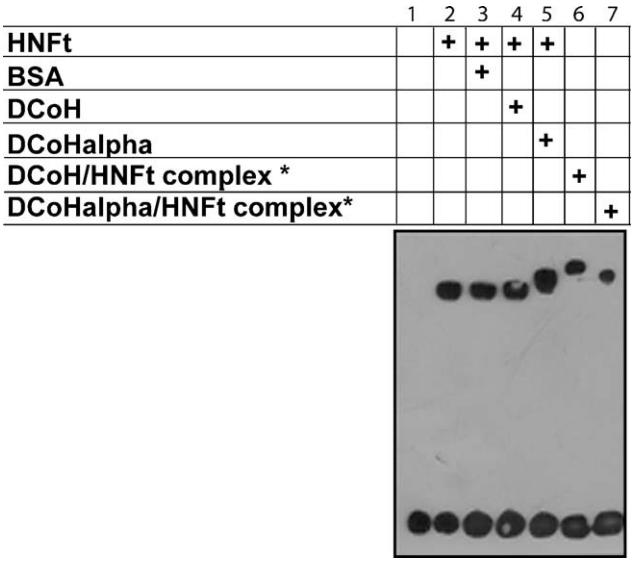

Fig. 5. Complex formation of DCoHα with HNFt and DNA in vitro. Electromobility shift assays demonstrate that DCoHα (lane 5), but not DCoH (lane 4) can form a complex in vitro with HNFt and the HRE in ApoB DNA. DNA-binding reactions were performed with 150 nM labeled ApoB and 500 nM purified HNFt dimer in the absence (lane 2) or presence (lane 3) of BSA (negative control), 150 nM DCoH (lane 4) or 150 nM DCoHα (lane 5) (calculated as the tetramer), purified DCoH/HNFt (lane 6, 38 nM functional heterotetramer), or DCoHα/HNFt (lane 7, 50 nM functional heterotetramer). The *DCoH/HNFt (lane 6) and *DCoHα/HNFt (lane 7), were produced by co-expression in *E. coli* and were run as migration controls. Lane 1 is labeled DNA (HRE from ApoB) alone. The figure is representative of three separate experiments (run time 50 min at 80 V). Gels were run for a longer time than in Fig. 3 to show the differences in migration distances.

quantitative EMSAs to measure DNA binding as a function of HNFt concentration. As Fig. 6 demonstrates, the addition of DCoHα, but not BSA or DCoH, to DNA-binding reactions results in a shift of the HNFt/DNA-binding isotherm indicative of increased DNA binding. Thus, not only does DCoHα form a stable complex with HNFt, but it results in a quantitative change in the ability of HNFt to interact with the cognate DNA sequence. Moreover, this occurs at a physiologically relevant concentration of HNFt (Fig. 6).

These results demonstrate the ability of DCoHα to alter HNF1-dependent DNA-binding in vitro, a prerequisite property to performing the transcriptional co-activator function of DCoH/DCoHα.

Catalytic activity of dehydratase/DCoH and dehydratase/DCoHα

We have previously measured the catalytic activity of purified human dehydratase/DCoH directly by a spectrophotometric assay and found that at the concentration in human liver (4–6 μM) the catalytic efficiency of the enzyme is sufficient to maintain a supply of tetrahydrobiopterin for phenylalanine hydroxylase during a phenylalanine load [16,19]. The catalytic properties of purified dehydratase/DCoHα were measured by the same procedure in parallel with dehydratase/DCoH so that a direct comparison of the activities of the two enzymes could be made. Kinetic parameters for each of the dehydratase forms were determined under conditions in which a linear relationship

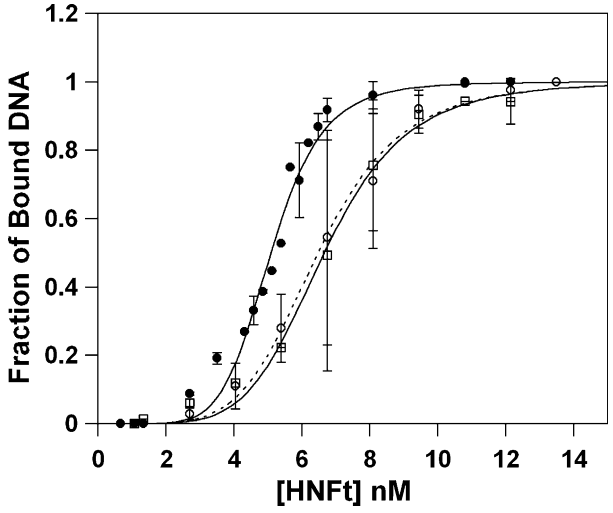

Fig. 6. Effect of DCoH and DCoHα on the binding of HNFt to the ApoB HRE. The binding of HNFt to DNA (ApoB HRE) in vitro is increased by DCoHα but not by DCoH which gives the same result as addition of BSA. One hundred and twenty-five nanomolar of either DCoHα (closed circles) or DCoH (open circles) (calculated as tetramer), or BSA (open squares) were included in the DNA-binding reactions. Data ($N = 3$ for each curve) were fitted to the Hill equation.

between protein concentration and V_{max} was found. DCoHα demonstrated significant dehydratase activity. The V_{max} (turnover) of dehydratase/DCoHα was 2–3 times

Table 1
Kinetic parameters for different dehydratase forms[a]

Protein	V_{max} (s^{-1})[b]	K_m (μM)	V_{max}/K_m (μM s^{-1})[c]
DCoH	10 ± 0.2	1.2 ± 0.2	8.3
DCoHα	24 ± 3	3 ± 0.5	8
DCoH/HNFt	10 ± 0.2	1.2 ± 0.2	8.3
DCoH/HNFt/DNA[d]	10 ± 0.2	1.2 ± 0.2	8.3
DCoHα/HNFt	10 ± 1	1.2 ± 0.3	8.3
DCoHα/HNFt/DNA[d]	10 ± 1	1.2 ± 0.3	8.3

[a] Dehydratase catalytic activity was measured in vitro with purified enzymes using a direct spectrophotometric assay with synthetic 4a-hydroxy-6(*S*)-methyltetrahydropterin as substrate. Reactions were performed at 10 °C in 25 mM BES-Na, pH 7.4.

[b] V_{max} is expressed as turnover number, i.e., nmol substrate dehydrated per nmol enzyme subunit per second.

[c] Catalytic efficiency.

[d] Reactions contained 45 nM DCoH/HNFt or DCoHα/HNFt, and 56–224 nM ApoB duplex DNA. All reactions were run in replicate (at least 6 times).

higher than that of dehydratase/DCoH. However, K_m was also 2–3 times higher, so that the catalytic efficiency (V_{max}/K_m) was the same for the two isozymes (Table 1).

Complexation of dehydratase/DCoH with HNFt had no effect on dehydratase activity. The concentration of dehydratase/DCoH in the liver is around three orders of magnitude higher than HNF1. Thus, whether or not complexation with HNFt has any effect on dehydratase activity is of little physiological consequence. On the other hand, complexation of HNFt with dehydratase/DCoHα resulted in a decrease in K_m and V_{max} to values that were the same as for dehydratase/DCoH. This change in kinetic parameters is suggestive of a conformational change of DCoHα when complexed with HNFt which is not apparent with dehydratase/DCoH. Addition of DNA duplex containing an HNFt response element (HRE) had no further effect on V_{max} or K_m (Table 1). Typically, the concentration of dehydratase in an enzymatic assay is 50–100 nM (based on subunit concentration). Even taking into account that there is ~10-fold dilution of protein during gel filtration chromatography, the concentration in a dehydratase activity assay is lower than in the 2 μM chromatogram in Fig. 2, which clearly shows that most of the protein is dissociated. Thus, one can conclude that the tetrameric form of the protein is not required for dehydratase activity. Furthermore, DCoHα appeared to be as stable as DCoH, showing no decrease in activity upon dilution and storage at 4 °C.

These results show that dehydratase/DCoHα, has the kinetic properties necessary for regenerating tetrahydrobiopterin cofactor for phenylalanine hydroxylase.

Discussion

Dehydratase isolated from human, rat, and bovine liver is the DCoH isozyme, as verified by mass spectrometry of the purified protein [18,24]. The concentration in the liver is in the range of 4–6 μM [17,19]. This high concentration is required to maintain a supply of tetrahydrobiopterin for phenylalanine hydroxylase during a phenylalanine load [16,17]. The cofactor product of the reaction, 4a-hydroxytetrahydrobiopterin, is unstable and when dehydratase is deficient rearranges to 7-biopterin. In normal individuals the activity of dehydratase in the liver is sufficient to maintain >98% of tetrahydrobiopterin in the fully reduced form [16], and thus 7-biopterin is not detected in the urine. In contrast, when dehydratase activity is non-optimal, as in human fetal liver [17], or in individuals with mutations in the dehydratase gene [10–12], 7-biopterin accumulates due to rearrangement of 4a-hydroxytetrahydrobiopterin. These patients are also hyperphenylalaninemic and excrete high concentrations of neopterin. Tetrahydrobiopterin regulates its own biosynthesis by feed-back inhibition of GTP cyclohydrolase, the first enzyme in its biosynthetic pathway. This inhibition is released by phenylalanine [25]. The high urinary neopterin observed in these hyperphenylalaninemic patients is formed from the product of GTP cyclohydrolase, and is a consequence of the release of feed-back inhibition by the elevated levels of phenylalanine. Thus, as phenylalanine levels are decreased neopterin production is also decreased. Although 7-biopterin excretion persists, the hyperphenylalaninemia and excretion of neopterin are transient. Patients with hyperphenylalaninemia are routinely treated with a phenylalanine restricted diet and in some cases also with tetrahydrobiopterin. The time at which phenylalanine levels are adequately decreased and therapy can be stopped is extremely variable ranging from a few months to greater than 5 years of age [10,26]. This is not inconsistent with our hypothesis that the transient nature of hyperphenylalaninemia is due to induction of DCoHα, since the time course and severity of symptoms would depend on the rate and level of induction, which could vary among individuals. However, even in the mildest cases, compensation by DCoHα is not sufficient to completely recycle tetrahydrobiopterin, since the excretion of 7-biopterin by these patients is persistent.

The concentration of HNF1 in liver is in the nanomolar range [27,28]. As our results show, the binding of DCoH and DCoHα to HNFt is extremely tight. Therefore, concentrations of DCoH or DCoHα in the nanomolar range would likely be adequate to perform the transcriptional coactivator functions of these proteins. Low levels of DCoHα protein have been detected in mouse liver [15], and EST libraries [15,29] and DNA chip analyses (survey using NCBI Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/; Accession Nos. GDS596 and GDS594) have revealed the presence of cDNA for DCoHα in mouse and human liver. However, the symptoms of patients with mutations in the dehydratase/DCoH gene indicate that the DCoHα isozyme is normally at a very low level, sufficient for transcriptional co-activator function but not for the recycling of tetrahydrobiopterin.

DCoH knockout mice [15] have high levels of 7-biopterin, neopterin, and phenylalanine as do humans with mutations in the dehydrates/DCoH gene. However, at the time that the mice were examined, all of these biochemical parameters were found to still be present [15], indicating

that DCoHα had not yet been induced to a level that could supply sufficient tetrahydrobiopterin for phenylalanine hydroxylation. This could be due to a lower initial level of DCoHα in mice than in humans, and/or the inability for DCoHα to be induced to sufficient levels in mice. A lower activity of the DCoHα isozyme in mice is also indicated by the disruption in transcriptional coactivator function in the DCoH knockout. These mice have cataracts and are glucose intolerant, symptoms which are not observed in humans with non-functional dehydratase/DCoH. The production of a DCoHα knockout mouse would allow the relative importance of DCoH and DCoHα to be determined, and may also provide markers for screening for mutations in the DCoHα gene in humans.

Transcriptional co-activator properties of DCoH and DCoHα

The transcription factor, HNF1, is involved in regulating the transcription of a large number of genes [6,7]. The ability of DCoH to form a complex with HNF1 in vivo was first reported by Crabtree and co-workers who purified the stable complex from rat livers and also demonstrated a DCoH-dependent 200-fold increase in HNF1-dependent transcription in cell culture [2]. Separate transient transfection studies have shown that DCoHα transfected in cells in culture can coactivate transcription in human cell lines through an HRE located in the fibrinogen promoter [14] although no quantitative comparison of the two coactivators was performed. A puzzling observation has been that humans with a form of hyperphenylalaninemia resulting from mutations in the gene for dehydratase/DCoH have no obvious symptoms of defects in the products of any HNF1-regulated genes. For example, apolipoprotein B transcription is regulated by a variety of transcription factors, including HNF1 which binds to an enhancer element located in the second intron. Loss of HNF1 binding resulted in an 80% reduction in the activity of the ApoB enhancer in transient transfection experiments [21]. However, patients with defective dehydratase/DCoH have no obvious symptoms of apolipoprotein B deficiency. One mutation found in patients is a truncation which would produce a protein with only 26 amino acids, ruling out the possibility of any remaining DCoH function in proteins lacking enzyme activity. Our data demonstrate that DCoHα has the ability to quantitatively enhance HNFt binding to the HRE of the Apolipoprotein B gene in vitro.

Although DCoH and DCoHα share 60% amino acid identity, and the crystal structures of the proteins show very little in the way of interfacial differences [30], the oligomeric states of the two proteins are dramatically different. In vitro, DCoH exists as a stable tetramer (Fig. 2) [18] yet the heterocomplex with HNFt contains only two subunits of DCoH [2]. On the other hand, DCoHα exists primarily as the dimer, and as our experiments show (Fig. 5), readily forms a complex with HNFt in vitro. Thus, the two dehydratase isoforms are not equivalent in terms of their potential for regulation of the transcriptional co-activator activity. While, at normal physiological concentrations, DCoHα, due to its dimeric state, is poised to interact with HNF1 directly and can quantitatively alter DNA binding (Fig. 6), interaction of DCoH with HNF1 would be regulated by either co-translational folding [2] or a cell-catalyzed event that alters the oligomeric state of the protein. The concentration of HNF1 calculated to be in liver is in the range of 5–10 nM [27,28]. The effect of DCoHα on the binding of HNFt and the sigmoidal nature of the DNA binding curves in this concentration range (Fig. 6) suggest that regulating dimerization of HNF1 is likely a physiologically relevant process. Thus, even at low concentrations, DCoHα may be sufficient to support normal HNF1-dependent transcription in humans, in the absence of functional DCoH.

Catalytic activity of dehydratase/DCoH and dehydratase/DCoHα

As an enzyme DCoH plays an essential role in maintaining levels of reduced tetrahydrobiopterin, especially in the liver under a load of phenylalanine. This is evident from the hyperphenylalaninemia (HPA) observed in newborns with defects in the dehydratase/DCoH gene (*PCBD*) [10]. Normal activity is adequate to maintain the levels of 4a-hydroxytetrahydrobiopterin to less than ~2% of the total liver pool [16,19]. By keeping the 4a-hydroxytetrahydrobiopterin pool low, the formation of 7-substituted pterins is decreased [10]. Interestingly, HPA caused by dehydratase/DCoH deficiency in newborns is transient, even though some of the naturally occurring mutations result in expression of only the first 26 amino acids, or in point mutations having less than 5% of wild-type activity [10]. This paradox would be resolved if the DCoHα isozyme possessed the necessary properties to fulfill the function of the dehydratase in these patients.

As shown in Fig. 2, DCoHα dissociates into dimers upon dilution, while DCoH is a stable tetramer. To relate these data to the dehydratase activity of DCoHα, the concentration of protein eluting from the size exclusion chromatography column was compared to the various concentrations of DCoHα used in the dehydratase activity assays. Typically, the concentration of dehydratase in an enzymatic assay is 50–100 nM (based on subunit concentration). Even taking into account that there is ~10-fold dilution of protein during the chromatography, the concentration in a dehydratase activity assay is lower than in the 2 μM chromatogram in Fig. 2, which clearly shows that most of the protein is dissociated. Thus, one can conclude that the tetrameric form of the protein is not required for dehydratase activity. Since the dehydratase enzymes appear to be catalytically functional regardless of whether they exist as dimers or tetramers, the inherent difference in oligomeric state as it relates to the cytosolic function of DCoH and DCoHα appears to be irrelevant.

Kinetic analyses demonstrated that the catalytic efficiency of dehydratase/DCoHα measured in vitro was equivalent to that of dehydratase/DCoH (Table 1). This would suggest that DCoHα could fully complement the inefficiencies of mutant DCoH enzyme activity in HPA afflicted individuals if it were expressed at high enough concentrations and in the correct location. In individuals born with enzymatically ineffective dehydratase/DCoH, the transient nature of the hyperphenylalaninemia could be explained by a lag in human dehydratase/DCoHα expression in the liver, which may be induced in response to dehydratase/DCoH deficiency.

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Supplementary data

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