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Assignment of Function to Histidines 260 and 298 by Engineering the E1 Component of the *Escherichia coli* 2-Oxoglutarate Dehydrogenase Complex; Substitutions That Lead to Acceptance of Substrates Lacking the 5-Carboxyl Group

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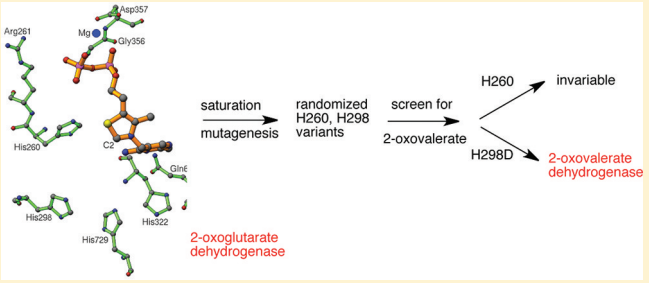
Supporting Information

ABSTRACT: The first component (E1o) of the *Escherichia coli* 2-oxoglutarate dehydrogenase complex (OGDHc) was engineered to accept substrates lacking the 5-carboxylate group by subjecting H260 and H298 to saturation mutagenesis. Apparently, H260 is required for substrate recognition, but H298 could be replaced with hydrophobic residues of similar molecular volume. To interrogate whether the second component would allow synthesis of acyl-coenzyme A derivatives, hybrid complexes consisting of recombinant components of OGDHc (o) and pyruvate dehydrogenase (p) enzymes were constructed, suggesting that a different component is the "gatekeeper" for specificity for these two multienzyme complexes in bacteria, E1p for pyruvate but E2o for 2-oxoglutarate.



In this work, we are interested in elucidating the factors that govern the specificity of the 2-oxoglutarate dehydrogenase multienzyme complex (OGDHc) toward its 5-carboxyl substituent, with the goal of synthesizing acyl-coenzyme A analogues, comprising a large class of metabolically relevant compounds participating in many metabolic pathways. The OGDHc catalyzes the rate-limiting step in the citric acid cycle,[1,2] which is the common pathway for the oxidation of fuel molecules, including carbohydrates, fatty acids, and amino acids, and catalyzes the formation of succinyl-coenzyme A (succinyl-CoA) according to eq 1.

$$\text{2-oxoglutarate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{succinyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (1)$$

The OGDHc is composed of multiple copies of three components:[3−6] (1) thiamin diphosphate (ThDP)-dependent 2-oxoglutarate dehydrogenase (E1o, EC 1.2.4.2), (2) dihydrolipoylsuccinyl transferase (E2o, EC 2.3.1.6), and (3) dihydrolipoyl dehydrogenase (E3, EC 1.8.1.4). The first two components carry out the principal reactions for succinyl-CoA formation, while the third reoxidizes dihydrolipoamide E2 to lipoamide E2. This mechanism is similar to those of other 2-oxoacid dehydrogenase complexes, including pyruvate dehydrogenase (PDHc) and branched-chain 2-oxoacid dehydrogenase. According to the X-ray structure of E1o, there were three His residues (H260, H298, and H729) positioned near the thiazolium ring of ThDP[3] (Figure 1), and substitution of H260 and H298 with Ala drastically reduced the activity. The results suggested that the histidine side chains interacted with the distal carboxylate of 2-oxoglutarate (2-OG).[3]

We constructed saturation mutagenesis libraries of H260, H298, H260/H298 and screened for activity toward 2-OG and an unnatural substrate, 2-oxovalerate (2-OV), in which a nonpolar methyl group replaces the charged carboxylate. Several E1o variants were isolated, some with the ability to decarboxylate 2-OV. The E1o variants created by the H260 and H298 substitutions were shown to be functionally competent according to their ability to produce a ThDP-bound predecarboxylation intermediate.[7] Next, we wished to determine whether the second E2o component would allow synthesis of acyl-coenzyme A derivatives. Hybrid complexes consisting of recombinant components of the *Escherichia coli* 2-oxoglutarate (o) and pyruvate dehydrogenase (p) enzymes were constructed (the E3 component is common to both), and it was demonstrated that the pyruvate dehydrogenase component (E1p) imparts specificity for acetyl-CoA formation

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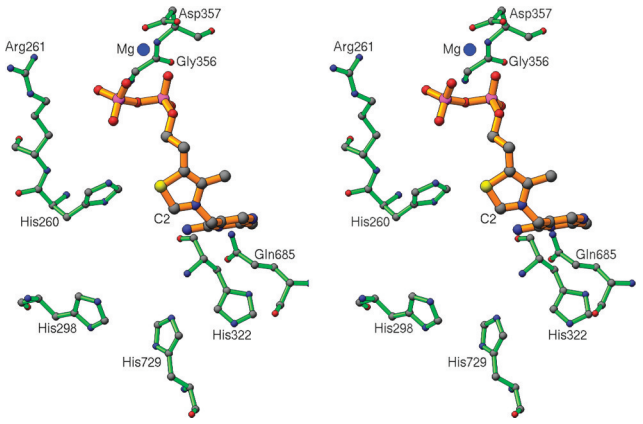

Figure 1. Stereoview showing histidines and a few other residues near the active site of the *E. coli* 2-oxoglutarate dehydrogenase multienzyme complex E1 component, showing their proximity to the reactive center C2 atom on thiamin diphosphate (ThDP). Coordinates for the protein atoms were obtained from Protein Data Bank (PDB) entry 2JGD,[3] but there were no coordinates for ThDP. The ThDP coordinates added were obtained by least-squares superposition of the active site area in the *E. coli* pyruvate dehydrogenase multienzyme complex E1 component (PDB entry 2IEA) with that of the same area in the reported apo structure (2JGD). This figure shows it is in roughly the same orientation as in Figure 3a of ref 3, with the added ThDP nearly identical in conformation and position to that shown in ref 3.

from pyruvate but E2o controls specificity for succinyl-CoA formation by OGDHc in Gram-negative bacteria.

Results of screening revealed that of the 352 colonies screened for H260 substitutions, seven were found to be positive with 2-OV and 61 with 2-OG.

Of the seven colonies found to be positive for 2-OV, DNA sequencing revealed that all were wild-type E1o. Of the eight colonies found to be positive with 2-OG, seven were identified as wild-type E1o and one was H260E. This immediately suggests that H260 is crucial for substrate binding. At position H298, 440 colonies were screened for 2-OG activity and 350 for 2-OV activity. DNA sequencing identified the H298T and H298L substitutions as being active with 2-OG and the H298D and H298V substitutions with 2-OV. Screening for dual H260/H298 substitutions with 2-OG (1232 colonies) and with 2-OV (1672 colonies) revealed several active variants: H260/H298T (with 2-OG), H260/H298D, H260/H298T, and H260E/H298N (with 2-OV).

The E1-specific activity was unaffected when E1o was reconstituted into E1o–E2o–E3 or E1o–E2p–E3 complexes. Similar E1-specific activity was found with 2-OG, pyruvate, or 2-OV using E1o by itself or assembled in the OGDHc or hybrid (E1o–E2p–E3) complex (Table 1, top). The activity of E1o was 24% toward pyruvate and 19% toward 2-OV compared to 2-OG. The E1-specific activity in the complex reconstituted from E1o and the E2o and E3 components (36% with pyruvate and 21% with 2-OV) remained similar to that with E1o by itself. Reconstitution of E1o in the hybrid complex with the dihydrolipoylacetyl transferase component (E2p) and E3 led to an E1-specific activity of 34% with pyruvate and 23% with 2-OV. This indicated that assembly into the OGDHc or hybrid complex does not affect significantly the E1-specific rates (Table 1, top). These results gave important evidence that pyruvate and 2-OV were substrates for E1o, as the 2,6-dichlorophenolindophenol (DCPIP) reduction assay clearly indicates that decarboxylation has taken place. No overall activity was detected with pyruvate or 2-OV for either the OGDHc or the hybrid E1o–E2p–E3 complex. The E1o–E2p–E3 hybrid complex exhibited detectable activity (2.2%) with 2-OG. In contrast to E1o, E1p exhibited activity only with pyruvate. In the DCPIP assay, E1p by itself showed no activity toward 2-OG or 2-OV. Furthermore, there was no activity for 2-OG or 2-OV for E1p reconstituted with either E2p and E3 or E2o and E3. Similar results were obtained in the overall activity assay (Table 1, bottom).

To provide further evidence that pyruvate is indeed a substrate for E1o, we conducted the following studies. (1) The carboligase side reactions commonly accompany ThDP-catalyzed decarboxylations. These reactions involve nucleophilic addition of the enamine (Scheme S1, Supporting Information) to the carbonyl carbon of the reactant or product, resulting in the formation of acetoin-like or acetolactate-like ligated products. Observation of carboligase products provides strong confirmation that decarboxylation of substrate had taken place. Upon addition of pyruvate to E1o, a negative CD band developed at 300 nm, indicating formation of optically active (*R*)-acetolactate (Figure S1, Supporting Information). The negative circular dichroism (CD) band at 300 nm was still present after removal of protein (not shown); both the sign of the band and its location are similar to those observed with the E636A variant of E1p (ref 8, confirmed for that enzyme by both CD and NMR). (2) It was next demonstrated that the pyruvate decarboxylated by E1o could reductively acetylate the lipoyl domain derived from E2p (LD-E2p), which could be detected

Table 1. E1-Specific and Complex Activity for E1o (top) and E1p (bottom)

	DCPIP activity (μmol min^{-1} mg^{-1})			overall activity [μmol min^{-1} (mg of E1o)$^{-1}$]	
substrate	E1o	E1o–E2o–E3	E1o–E2p–E3	E1o–E2o–E3	E1o–E2p–E3
2-OG (2 mM)	0.34 ± 0.01 (100%)	0.37 ± 0.02 (109%)	0.40 ± 0.01 (119%)	16 ± 0.4 (100%)	0.36 ± 0.01 (2.2%)[a]
pyruvate (25 mM)	0.08 ± 0.01 (24%)	0.12 ± 0.01 (36%)	0.12 ± 0.01 (34%)	no activity	no activity
2-OV (25 mM)	0.06 ± 0.001 (19%)	0.07 ± 0.03 (21%)	0.08 ± 0.01 (23%)	no activity	no activity
detected by FTMS		not measured		succinyl-CoA (2-OG)	succinyl-CoA[b] (2-OG)
	DCPIP activity (μmol min^{-1} mg^{-1})			overall activity [μmol min^{-1} (mg of E1p)$^{-1}$]	
substrate	E1p	E1p–E2p–E3	E1p–E2o–E3	E1p–E2p–E3	E1p–E2o–E3
pyruvate (2 mM)	0.75 ± 0.01 (100%)	0.64 ± 0.07 (85%)	0.70 ± 0.02 (93%)	28 ± 1.0 (100%)	no activity
2-OG,[c] 2-OV[d]	no activity	no activity	no activity	no activity	no activity

[a]The lowest activity detected was 0.068 ± 0.004 mmol min^{-1} (mg of E1o)$^{-1}$. [b]Apparently, even 2.2% could be detected by FTMS, confirming a low percentage activity. [c]At 2 mM. [d]At 45 mM.

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Table 2. Effects of H298 and H260/H298 Substitutions on the E1-Specific Activity of OGDHc

(A) 2-OG				
substitution	DCPIP activity (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_m ($\times 10^{-3}$ mM)	k_{cat}/K_m (s^{-1} mM^{-1})
none	0.620 ± 0.03[a]	2.15 ± 0.10	2.61 ± 0.376	824
H298L	0.120 ± 0.028	0.415 ± 0.098	3.41 ± 0.237	122
H298T	0.0018 ± 0.0001	0.0064 ± 0.0002	4.25 ± 0.160	1.5
H298D	nd[b]	nd[b]	nd[b]	nd[b]
H298V	0.029 ± 0.003	0.099 ± 0.009	165 ± 6	0.60
H260E/H298N	0.039 ± 0.004	0.134 ± 0.013	2.73 ± 0.469	49.1
H260E	0.0091 ± 0.0007	0.032 ± 0.003	383 ± 35.5	0.0084
(B) 2-OV				
substitution	DCPIP activity (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
none	0.022 ± 0.002	0.076 ± 0.007	16.3 ± 4.00	0.0047
H298L	0.027 ± 0.003	0.094 ± 0.011	6.30 ± 0.64	0.015
H298T	0.0086 ± 0.0009	0.030 ± 0.003	15.3 ± 1.00	0.0020
H298D	0.357 ± 0.018	1.24 ± 0.062	7.02 ± 0.023	0.18
H298V	0.160 ± 0.005	0.556 ± 0.018	8.96 ± 0.005	0.062

[a]E1o was from a different preparation than that in Table 1. [b]Not detectable above the background.

by Fourier transform mass spectrometry (FTMS). The rate constant for reductive acetylation of LD-E2p by E1o and pyruvate was 0.0056 ± 0.001 s^{-1}, compared to 51.7 ± 5.4 s^{-1} for E1p and pyruvate under the same conditions. The reductive acetylation of the didomain comprising lipoyl and subunit binding domains of the E2p was also demonstrated (not shown). Formation of succinyl-CoA by the E1o−E2o−E3 complex and 2-OG was confirmed by both the isotopic pattern and m/z ratio (868.14) of a succinyl-CoA standard via FTMS (Figure S2, Supporting Information). Next, the E1o−E2p−E3 complex was reacted with 2-OG to produce detectable amounts of succinyl-CoA (Table 1, top), displaying an isotopic distribution similar to that of the standard spectrum of succinyl-CoA (Figure S3, Supporting Information).

These additional experiments were conducted, in part, to also address the finding by Frey's group that in the OGDHc isolated from *E. coli*, there is indeed as much as 10% E1p,[9] whose presence would confound our interpretation. We needed to demonstrate that the pyruvate decarboxylating activity displayed by E1o was not an artifact of the presence of E1p. Our experiments described above, and the fact that all of the components of OGDHc were His-tagged and independently overexpressed, rule out any significant contamination by intrinsic E1p components.

Next, the effect of H260 and H298 substitutions on E1o activity was examined. Saturation mutagenesis data revealed that H298 could tolerate substitution. The DCPIP activity for the E1o variants with 2-OG ranged from <1% (H298T) to 19% (H298L) (Table 2A). The K_m for 2-OG increased for some E1o variants, while the catalytic efficiency (k_{cat}/K_m) of all variants with 2-OG was lowered. The catalytic efficiency for the best E1o variants decreased ∼7-fold for H298L and ∼17-fold for H260E/H298N and was severely compromised for other variants (Table 2A). Remarkably, the H298D and H298V substitutions converted E1o to 2-oxovalerate dehydrogenase with activities comparable to that observed with 2-OG (Table 2B). The H298 substitution in E1o also affected the overall OGDHc activity (Table S1, Supporting Information). The relative activities according to the E1-specific and overall activity assays were approximately paralleled. Finally, OGDHc did not show any overall activity toward 2-OV, again implying discrimination at the E2o level.

CD experiments revealed formation of a predecarboxylation intermediate analogue between E1o and 2-oxophosphonate and 2-oxophosphinate analogues. The Rutgers group has published extensive reports of CD detection of ThDP-bound covalent intermediates on enzymes with substrate mimics derived from methyl acetylphosphonate (MAP) and acetylphosphinate (AcP^-), which are analogues of pyruvate (Figure S4, Supporting Information).[7,10,11] It had been reported that succinyl phosphonate (SP^{2-}) and its monomethyl phosphonate ester ($SPME^-$), which are analogues of 2-OG, inhibit the partially purified OGDHc complex from brain with an $S_{0.5,SP^-}$ of 0.12 mM, which is in the range of $K_{M,2-OG}$ values of 0.1−0.2 mM reported for OGDHc from different sources (see the Supporting Information for synthesis of the substrate analogues).[12] The inhibitory effect of $SPME^-$ was also demonstrated for OGDHc from *E. coli* and pigeon breast muscle[13] and very recently for MenD.[14] First, E1o was titrated with AcP^- because this analogue was found to bind strongly to a number of ThDP enzymes.[7] CD spectra of E1o titrated with AcP^- revealed the generation of two CD bands: a positive one at 297 nm earlier assigned to the 1′,4′-iminopyrimidine tautomer (IP) of the first covalent intermediate (predecarboxylation in Scheme S1 of the Supporting Information) and a negative one at 330 nm assigned to a Michaelis complex.[11] The calculated values of K_d for AcP^- were 0.32 mM (at 297 nm) and 0.31 mM (at 330 nm) (Figure S5A and Table S2, Supporting Information) compared with a $K_{M,2-OG}$ of 90 μM (Table S1, Supporting Information). Next, $SPME^-$, SP^{2-}, and propionyl phosphinate (PP^-), analogues of 2-OG and 2-OV, were evaluated in the CD experiment (Table S2 and Figure S5B,C, Supporting Information). These are the first CD experiments to demonstrate that upon addition of substrate analogues, E1o forms a tetrahedral ThDP-bound predecarboxylation intermediate analogue, resembling those formed from substrates. The K_d values determined are in the micromolar range ($SPME^-$ phosphonate monoester gives the best K_d of 10 μM, while the diacid SP^{2-} is approximately 3 times weaker), demonstrating that some of the tested compounds could be powerful inhibitors of the E1o component of OGDHc (Table S2, Supporting Information). Similar CD experiments with the H298 E1o variants of phosphonate and phosphinate analogues of 2-oxoacids (Figures S6−S9, Supporting Information) indicated that this substitution is not favorable for binding of $SPME^-$, excepting H298T. For the H260E/H298N variant, no

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CD band was detected at 300 nm. On the other hand, H298 substitutions and the double H260E/H298N substitution were favorable for binding of PP^-, which is a substrate analogue of 2-OV [the H298D variant has a K_d value of 9.6 μM as compared with a value of 39 μM for E1o (Figure S10 and Table S2, Supporting Information)]. In general, the K_{d,PP^-} values were smaller (binding was stronger) than that for E1o, and K_{d,PP^-} ranged from 5 to 22 μM (Figures S11–S14, Supporting Information).

The following could be concluded about the roles of His260 and His298 in E1o. The H260E, H298T, H298V, H260E/H298N, and H298L substitutions exhibited activity with 2-OG. The H298D and H298V substitutions led to an active enzyme with 2-OV, displaying improvement in k_{cat}/K_m in comparison to E1o. While finding a positively charged or hydrophilic side chain in its place could be anticipated, the most active variant, H298L, is unexpected, with a K_m comparable to that of E1o. Being only slightly larger than His, this substitution with Leu may only fulfill a volume constraint (the van der Waals volumes for Leu and His are 124 and 118 Å^3, respectively). A study of the active center residues of the ThDP enzyme benzoylformate decarboxylase led to a similar conclusion about residue His281: it could be replaced with Phe or Leu without significant activity loss.[15] Randomization at His260 yielded only one E1o variant (H260E) with low activity toward 2-OG and much better activity toward 2-OV.

The active variants identified by the E1-specific assay were shown to be functionally competent according to their ability to form predecarboxylation covalent intermediate analogues between the ThDP and phosphono or phosphino analogues of 2-OG and 2-OV as judged by CD. The values of K_d calculated from CD data are in the micromolar range as compared with reported values of $K_{m,2-oxoglutarate}$ (0.1–0.2 mM)[12] and point to increasing binding potency with increasing chain length. For all H298 variants, PP^- was more firmly bound than $SPME^-$, suggesting conversion of function from 2-oxoglutarate dehydrogenase to 2-oxovalerate dehydrogenase, especially in the H298D and H298V variants, which display relatively high activity with 2-OV. The single low-activity H260E E1o variant did not display a measurable CD signal with PP^- or $SPME^-$, consistent with kinetic analysis. The randomization experiment, kinetic study, and CD detection of covalent intermediate analogues provide strong evidence that H260 is crucial and indispensible for 2-OG recognition.

Surprisingly, the E1o could decarboxylate 2-OV and pyruvate, in addition to 2-OG, according to the DCPIP assay, an assay adequate for confirming decarboxylation of the substrates. To determine whether the second component would allow synthesis of acyl-coenzyme A derivatives from substrates accepted by the engineered E1o, hybrid complexes consisting of recombinant components of the *E. coli* OGDHc (o) and PDHc (p) were constructed (the E3 component is common to both). Upon reconstitution of E1o with E2o and E3 in OGDHc, the overall activity with 2-OG was 17 μmol min^{-1} (mg of E1o)$^{-1}$ and correlated well with recently published data for E1o.[3] No NADH production was detected with pyruvate or 2-OV in the E1o–E2o–E3 and E1o–E2p–E3 hybrid complexes. Detection of succinyl-CoA formation by mass spectrometry, and of reductively acetylated and succinylated LD-E2p, and of the activity of the reconstituted complex by a NADH kinetic assay allowed us to conclude that (1) the E1o–E2o–E3 and E1p–E2p–E3 complexes produced the respective acyl-CoA products and NADH, (2) the E1o–E2p–E3 complex could produce succinyl-CoA from 2-OG, (3) the E1p–E2o–E3 complex could not produce acetyl-CoA from pyruvate, and (4) E1o could reductively acetylate and reductively succinylate LD-E2p. Apparently, a different component is the "gatekeeper" for specificity for acyl-CoA formation by these two important multienzyme complexes in Gram-negative bacteria, E1p for pyruvate but E2o for 2-OG. The ability of E1o to reductively acetylate LD-E2p and the ability of the hybrid E1o–E2p–E3 complex to produce succinyl-CoA provide strong confirmation of this statement.

The principal difference between this and earlier work is that here recombinant individual components were used, while earlier work used isolated complexes, PDHc and OGDHc, or their subcomplexes. Notably, Frey and associates demonstrated that 10% E1p copurified with *E. coli* OGDHc; however, the overall activity was ~1% of that with PDHc, already suggesting that E2o also conferred substrate specificity in terms of rates.[9] deKok and co-workers used the OGDHc isolated from *Azotobacter vinelandii* and found modest overall activity with pyruvate, but no E1-specific activity was detected, in contrast to our findings.[16] "Promiscuous" substrate utilization has been identified in several ThDP enzymes: "engineering" by single-amino acid substitutions has been shown to lead to changes in both substrate and reaction specificity from a decarboxylase/dehydrogenase activity to carboligase-like activity in yeast pyruvate decarboxylase,[17] E1p,[8] and benzoylformate decarboxylase,[18,19] among others; the specificity of acetohydroxyacid synthase toward pyruvate as a donor has been attributed to hydrophobic residues.[20]

Our results rule out an acid–base or hydrogen bonding role for residue H298 but confirm a hydrogen bonding role for H260. Hence, to create complexes capable of accepting alternate 2-oxo acids, it will also be necessary to engineer the E2o active center.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and methods, Tables S1 and S2, Scheme S1, and Figures S1–S14. This material is available free of charge via the Internet at http://pubs.acs.org.

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