Published in final edited form as:
FEBS Lett. 2007 June 26; 581(16): 2988–2992.

Amino acid residues responsible for the recognition of dichloroacetate by pyruvate dehydrogenase kinase 2

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Abstract

Dichloroacetate (DCA) is a promising anticancer and antidiabetic compound targeting the mitochondrial pyruvate dehydrogenase kinase (PDHK). This study was undertaken in order to map the DCA-binding site of PDHK2. Here, we present evidence that R114, S83, I157 and, to some extent, H115 are essential for DCA binding. We also show that Y80 and D117 are required for the communication between the DCA-binding site and active site of PDHK2. These observations provide important insights into the mechanism of DCA action that may be useful for the design of new, more potent therapeutic compounds.

Keywords

Dichloroacetate; pyruvate dehydrogenase kinase; protein phosphorylation; mitochondria; oxidative decarboxylation of pyruvate; carbohydrate metabolism; cancer; diabetes

1. Introduction

Pyruvate dehydrogenase kinase (PDHK) is a dedicated mitochondrial protein kinase that regulates the enzymatic activity of pyruvate dehydrogenase multienzyme complex (PDC) [1] and, thereby, controls the rate of aerobic oxidation of carbohydrate fuels [2]. In mammals, there are at least four biochemically and genetically different forms of PDHK, *i.e*. PDHK1, PDHK2, PDHK3, and PDHK4 [3]. These isoforms are thought to be responsible for the tissue-specific regulation of PDC [4]. *In vitro*, the kinase activity is regulated by a variety of metabolites. The substrates of pyruvate dehydrogenase reaction (pyruvate, NAD^+, and CoA) act as PDHK inhibitors [1,3,4], whereas the products, *i.e*. NADH and acetyl-CoA, act as PDHK activators [1,3,4]. Thus, it is generally believed that under physiological conditions PDHK activity reflects the ratio of intramitochondrial concentrations of NAD^+/NADH and CoA/acetyl-CoA, as well as the intramitochondrial concentration of pyruvate. This rather complex regulation allows PDHK to adjust the activity of PDC according to the existing metabolic demand on a minute-to-minute basis.

Structurally, PDHK is a dimer of two identical subunits associated in tail-to-tail orientation [5,6,7]. Each PDHK subunit consists of two almost equal in size domains: the amino-terminal

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"regulatory" domain (R domain) and the carboxy-terminal "kinase" domain (K domain) [5, 7]. The R domain is assembled as a four helix bundle and carries the binding sites for pyruvate and its synthetic analog dichloroacetate (DCA), as well as for lipoyl-bearing domain (LBD) of PDC [6,7]. The K domain bears the nucleotide-binding site folded as a mixed α/β sandwich and also contributes to the LBD-binding site of the neighboring subunit [5,7]. The active site of PDHK is presumably located on the interface between the K and R domains [5,7].

Despite the recent progress made in structural characterization of PDHK, the molecular mechanisms responsible for the regulation of kinase activity remain poorly understood. The available structural data suggest that the allosteric control of PDHK activity requires communications over distances greater than 20 Å [6,7]. This makes it difficult to trace the exact signaling pathways involved. In addition, the majority of PDHK allosteric binding sites have not yet been characterized. Thus, this study was undertaken in order to map the pyruvate/dichloroacetate-binding site of PDHK2, which is thought to be located in the middle of the four helix bundle of the R domain [7]. Here, for the first time, we describe the structural requirements that largely define the affinity of PDHK2 for DCA.

2. Materials and methods

2.1. Vector construction and protein expression

Construction of the expression vectors for E1, E2/E3BP subcomplex, lipoyl-protein ligase A, and PDHK2 was described elsewhere [4,8,9,10]. Mutagenesis was conducted on previously described pPDHK2 vector using appropriate oligonucleotide primers [4]. Reactions were set up using the ExSite™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) essentially as recommended by the manufacturer. The presence of mutations and fidelity of the rest of the DNAs were confirmed by sequencing [11]. General conditions for the expression of E1, E2/E3BP subcomplex, and PDHK2 were described previously [4,8,9]. PDHK2 carrying various point mutations were expressed following the established protocol [4]. Purification of E1, E2/E3BP subcomplex, and of PDHK2 was described elsewhere [8,9]. The protein composition of each protein preparation was evaluated by SDS/PAGE analysis. Gels were stained with Coomassie R250. All preparations used in the present study were more than 90% pure.

2.2. PDHK2 activity assay

The activity of PDHK2 was determined based on the incorporation of [^{32}P] phosphate into E1 during 1 min of the reaction as described previously [4]. All assays received a negative control (minus PDHK2) to determine the non-specific incorporation and were conducted in triplicates. For DCA titration experiments, titrations with acetic acid were performed as controls. Both DCA and acetate were used as sodium salts.

2.3. Tryptophan fluorescence quenching

Steady state fluorescence spectra of PDHK2 were recorded at 20°C in 50 mM potassium phosphate buffer, pH 7.5, containing 0.25 mM EDTA and 2.0 mM $MgCl_2$, using ISS PC1 photon-counting spectrofluorometer equipped with an auto titrator (ISS, Inc., Champaign, Illinois) with a band-pass of 1 mm on both the excitation and the emission monochromators. Samples were excited at 290 nm and emission spectra were recorded from 320 to 420 nm. The titration experiments were conducted in standard (1×1 cm) cells containing protein samples of the initial volume of 2.0 ml. DCA was added in 2– or 10–μl increments with constant stirring. PDHK2 and its variants were used in the concentration of 1.0μM. Results were corrected for the non-specific fluorescence quenching determined in the presence of increasing concentrations of acetate. Corrected data were transformed and analyzed using approaches described by Hiromasa and colleagues [12].

2.4. Statistical analysis

The reported parameters represent the means ± S.D. obtained for three to five independent determinations. Statistical analysis was performed by an unpaired Student's *t*-test. $P<0.05$ was considered to be statistically significant.

2.5. Other procedures

SDS/PAGE was carried out according to Laemmli [13]. Protein concentrations were determined according to Lowry [14] with bovine serum albumin as a standard.

3. Results and discussion

3.1. Characterization of PDHK2 variants carrying amino acid substitutions within the putative DCA-binding pocket

DCA is a synthetic analog of pyruvate that inhibits PDHK activity acting in concert with ADP [3]. This compound shows a great potential as an oral antidiabetic agent reducing blood glucose and triglycerides without stimulating insulin secretion [15]. In addition, DCA very effectively decreases tumor growth *in vitro* and *in vivo* by shifting pyruvate metabolism from glycolysis and lactate production to glucose oxidation in the mitochondria [16]. Recent structural studies conducted by Knoechel and colleagues [7] identified DCA in a small pocket located in the middle of the four helix bundle of PDHK2 R domain. Whether this pocket represents a physiologically sound DCA-binding site has not been established.

This study was undertaken in order to examine the physiological significance of putative DCA-binding pocket identified by Knoechel *et al*. [7] and to establish the molecular mechanism responsible for the recognition of DCA by PDHK2. Toward this end, we carried out an alanine-scanning mutagenesis targeting the amino acid residues lining the DCA-binding pocket of rat PDHK2, *i.e*. L53, Y80, S83, I111, R112, R114, H115, D117, R154, I157, R158, and I161, respectively (Fig. 1).

Mutations were introduced in PDHK2 cDNA using oligonucleotide-directed mutagenesis. Mutagenized cDNAs were expressed in bacteria essentially as described elsewhere [4]. All cDNAs directed an abundant synthesis of corresponding protein products. Cellular fractionation revealed that all PDHK2 variants were expressed in soluble form. The yield of soluble PDHK-I157A was lower than that of other proteins due to it tendency to accumulate in inclusion bodies. Purification of soluble protein kinases was achieved using immobilized metal affinity chromatography [4].

The ability of mutant PDHK2 enzymes to phosphorylate pyruvate dehydrogenase was assessed in the standard phosphorylation assay (Fig. 2). With free E1 as a substrate, the majority of PDHK2 variants (PDHK2-L53A, PDHK2-Y80A, PDHK2-I111A, PDHK2-R112A, PDHK2-D117A, PDHK2-R158A, and PDHK2-I161A) displayed the activities comparable to that of the wild-type PDHK2, while the activities of PDHK2-S83A, PDHK2-R114A, and PDHK2-H115A were somewhat greater than the activity of wild-type kinase. The activity of PDHK2-I157A variant was significantly reduced. Similar experiments carried out in the presence of E2 revealed that all PDHK2 variants retained their sensitivity to the E2 component showing a several-fold increase in kinase activity in response to the addition of E2 (Fig. 2). Thus, almost all PDHK2 variants carrying amino acid substitutions within the putative DCA-binding pocket behaved similar to the wild-type enzyme suggesting that these mutations did not cause any functionally significant rearrangements in PDHK2 structure. PDHK2-I157A variant showed significantly reduced kinase activity, but responded to the addition of E2 similar to the wild-type enzyme.

3.2. Effects of amino acid substitutions within the putative DCA-binding pocket of PDHK2 on the DCA-dependent inhibition of kinase activity and DCA binding

The effects of DCA on enzymatic activities of mutant PDHK2 proteins were assayed over a wide range of DCA concentrations varied from 0.1μM to 100 mM (Fig. 3). As summarized in Table 1, a number of mutations, *i.e*. R112A, R154A, R158A, and I161A, had little if any effect on the ability of DCA to inhibit PDHK2 activity, while other mutations (L53A, I111A, and H115A) caused a rather modest, approximately 3- to 8-fold, increase in the apparent IC_{50} values for DCA. In contrast, four mutant kinases, *i.e*. PDHK2-Y80A, PDHK2-S83A, PDHK2-R114A, and PDHK2-D117A, did not show an appreciable decrease in activity when DCA in the assay mixture was varied from 0.1 to 10 mM (Fig. 3 and Table 1). For these proteins, we could not reach half-inhibition even at the concentration of DCA as high as 100 mM. Finally, PDHK2-I157A variant displayed a greatly reduced IC_{50} value for DCA (approximately 13μM). Taken together, these data strongly suggest that Y80, S83, R114, and D117 along with I157 are extremely important for the effect of DCA on PDHK2 activity.

In order to get further insight into the molecular mechanisms responsible for recognition of DCA by PDHK2, we investigated binding of DCA (varied from 0.1μM to 100 mM) to the wild-type and mutant PDHK2 proteins using the intrinsic tryptophan fluorescence quenching assay developed by Hiromasa and colleagues [12] (Fig. 4). In general agreement with the activity determinations above, R112A, R154A, R158A, and I161A variants bound DCA similarly or slightly better than the wild-type kinase, whereas the affinity of PDHK2-L53A, PDHK2-I111A and of PDHK2-H115A for DCA was approximately 3- to 5-fold worse than that of unaltered PDHK2 (Table 2). Furthermore, DCA did not cause an appreciable fluorescence quenching of either PDHK2-S83A or PDHK2-R114A variant and quenched fluorescence of PDHK2-I157A at a very low concentrations of the ligand (EC_{50} of approximately 3μM). This outcome is consistent with the interpretation that weak inhibition of PDHK2-S83A and PDHK2-R114A activities by DCA reflects poor DCA binding by these mutant kinases, while hypersensitivity of PDHK2-I157A kinase to DCA is a consequence of its greater affinity for DCA. In marked contrast with the activity assay, DCA effectively quenched the intrinsic fluorescence of PDHK2-D117A and PDHK2-Y80A proteins with EC_{50} values of 0.63μM, and 1.98μM, respectively (Table 2), indicating that these proteins were capable of DCA binding similarly or slightly worse than the wild-type kinase. These observations strongly suggest that neither Y80 nor D117 directly participate in DCA binding.

3.3. Recognition of DCA by PDHK2

Recent crystallographic studies identified DCA in the pocket located in the middle of PDHK2 R domain [7]. Based on structural considerations, the authors proposed that DCA is sandwiched between H115 and I157, while its carboxylate group forms a salt-bridge with R154. In addition, the authors hypothesized that R158 controls the access of DCA into the binding pocket [7]. In this study, we examined this model using alanine scanning mutagenesis. In agreement with the existing model we found that both H115 and I157 are essential for DCA binding. In contradiction with this model were our results showing that neither R154 nor R158 play any significant role in DCA recognition. Instead, S83 and R114 located on the opposite side of DCA-binding pocket were found to be crucial for DCA binding. The rational for this discrepancy is currently unknown.

Of particular interest appears to be the outcome of experiments with PDHK2-Y80A and PDHK2-D117A proteins. Both kinases bound DCA similarly to the wild-type PDHK2. However, neither of them displayed an appreciable decrease in activity in response to the addition of DCA indicating a lack of communication between the DCA-binding site and active site. Indeed, in PDHK2 structure these sites are more than 20 Å apart and, therefore, there should be some mechanism whereby the signal is relayed from one site to another. It seems

reasonable to propose that both Y80 and D117 are part of this signal transduction network connecting the DCA-binding and active sites of PDHK2.

Thus, although the results of this study provide the first evidence that DCA containing pocket identified by Knoechel and colleagues [7] represents a physiologically sound DCA-binding site in PDHK2, it is clear that additional structural studies are required to further refine our understanding of DCA recognition and signaling within PDHK2 molecule.

Acknowledgements

This work was supported by grant GM 51262 from the U.S. Public Health Services. The authors are thankful to Dr. Anthony Gatenby at DuPont Central Research and Development, Wilmington, DE for the plasmid directing the synthesis of molecular chaperonins GroEL and GroES.

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Abbreviations

PDC
: pyruvate dehydrogenase complex

PDHK
: pyruvate dehydrogenase kinase

PDHK1,PDHK2, PDHK3, and PDHK4
: isozymes 1, 2, 3, and 4 of pyruvate dehydrogenase kinase

E1
: pyruvate dehydrogenase component of PDC

E2
: dihydrolipoyl acetyltransferase component of PDC

E3
: dihydrolipoamide dehydrogenase component of PDC

DCA
: dichloroacetate

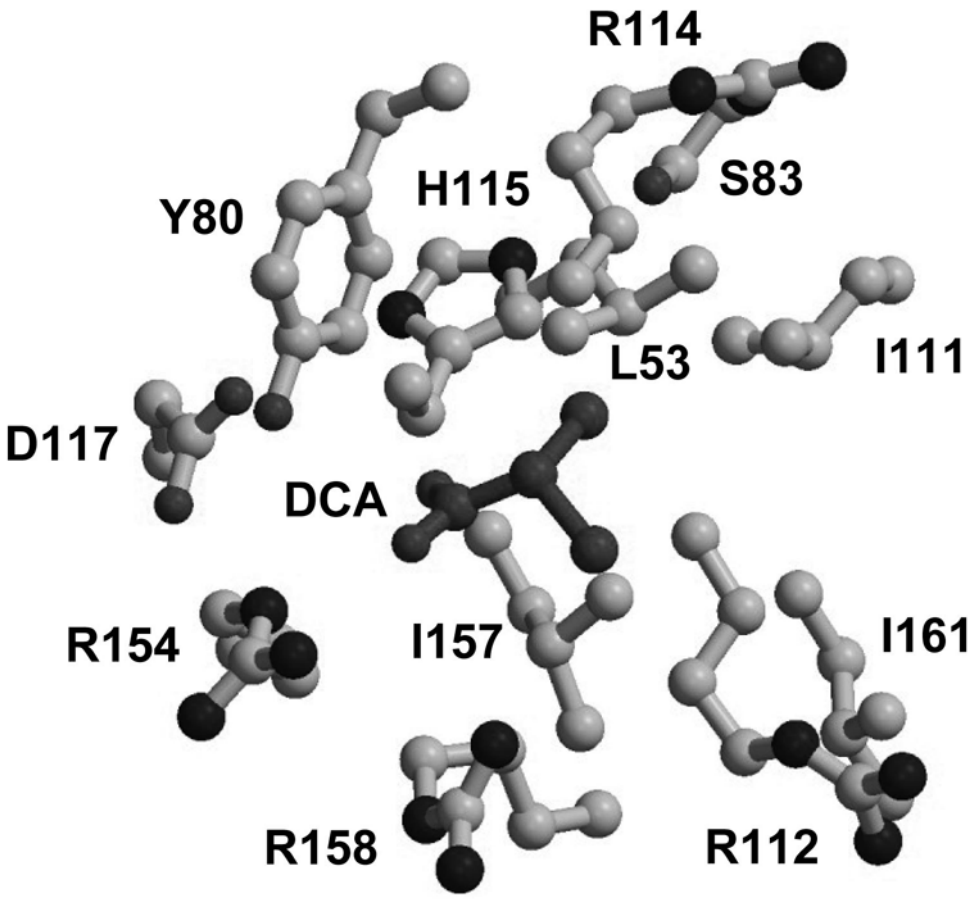

Figure 1. Putative DCA-binding site of PDHK2

Ball-and-stick representation of DCA-binding site of PDHK2 based on *2bu8* coordinates [7]. Shown are amino acid residues of PDHK2 located within contact distance from bound DCA. The graphics were generated using MolScript v2.1.2 and Raster3D v2.7 programs.

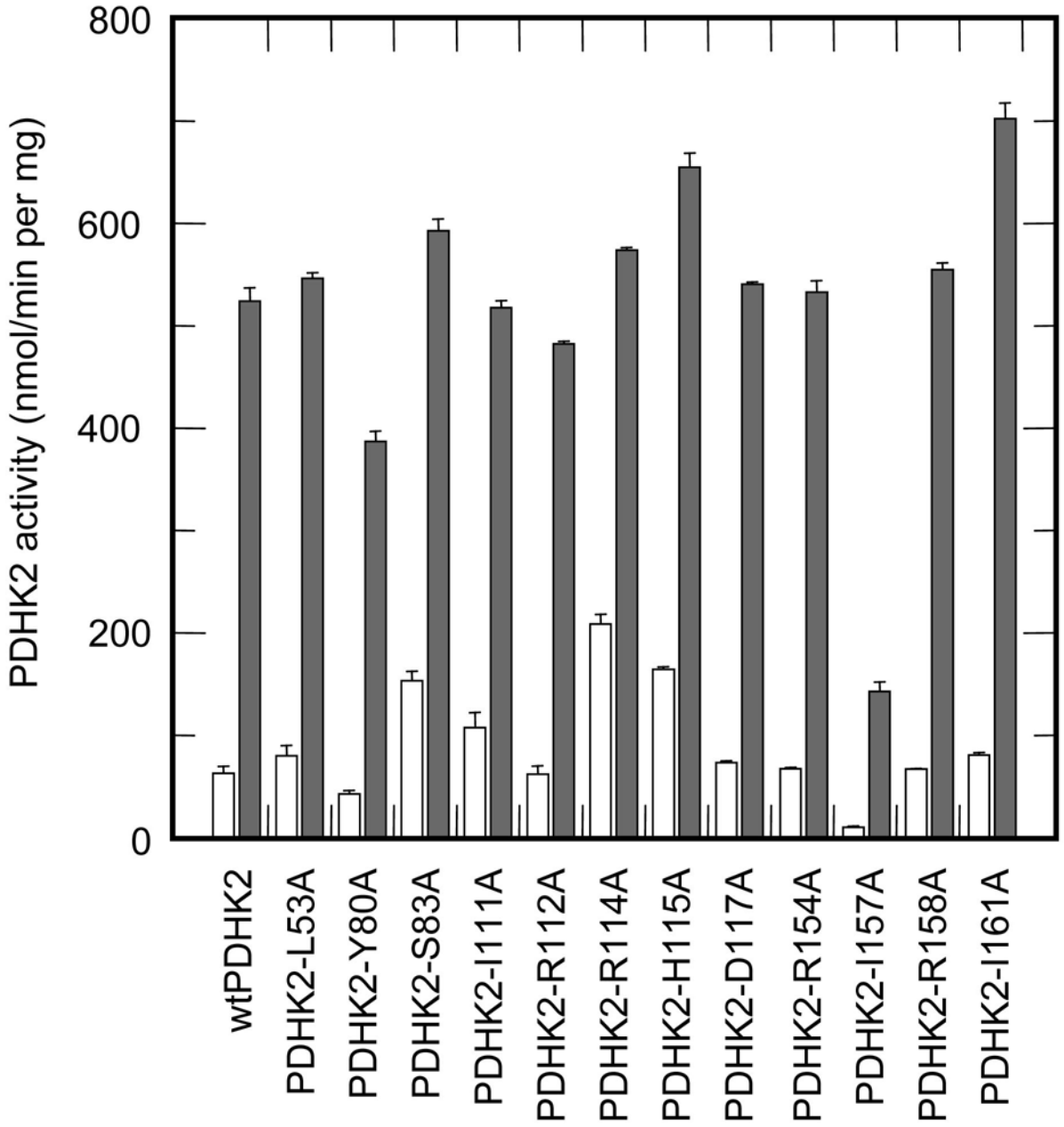

Figure 2. Enzymatic activities of PDHK2 proteins carrying point mutations within the putative DCA-binding site

Activity measurements were made as described under "Materials and methods" using 5μM E1, 20 nM PDHK2, and 200μM [γ-^{32}P]ATP with specific activity of 100–200 cpm/pmol (*open bars*) or 5μM E1, 20 nM E2, 20 nM PDHK2, and 200μM [γ-^{32}P]ATP (*closed bars*). All measurements were conducted with negative control (minus PDHK2) in order to determine the non-specific incorporation. Shown are the means ± S.D. obtained for three to five independent determinations.

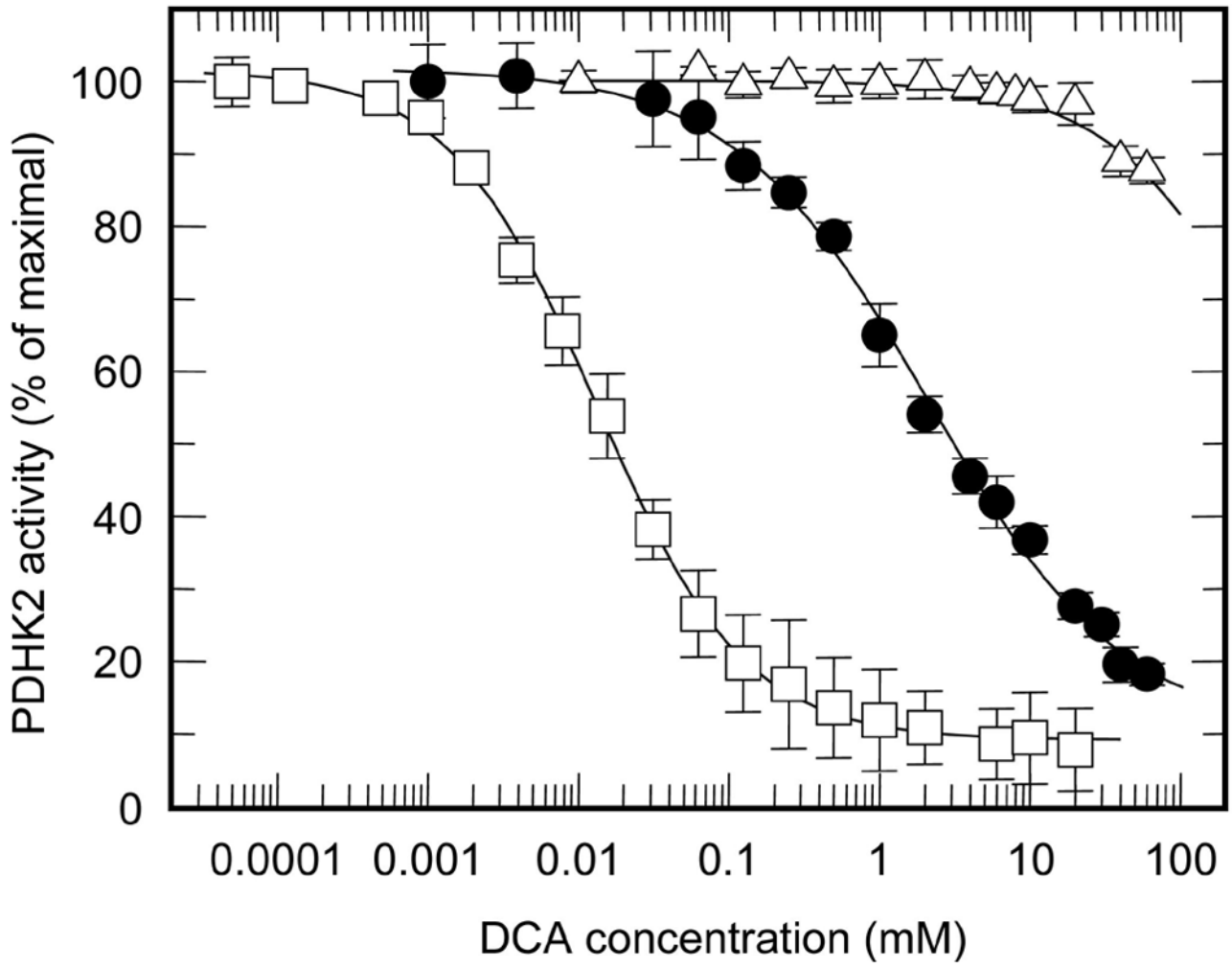

Figure 3. Inhibition of PDHK2 enzymatic activity by DCA

Incremental decreases in activity with increasing DCA concentration for wild-type PDHK2, PDHK2-I157A, and PDHK2-R114A are shown by *closed circles*, by *open squares*, and by *open triangles*, respectively. Activity measurements were made as described under "Materials and methods" using 5μM E1, 20 nM E2, 20 nM PDHK2, and 50μM [γ-^{32}P]ATP (specific activity 100–200 cpm/pmol). The results were corrected for non-specific inhibition determined in the presence of increasing concentration of acetate. Background-corrected data were fitted using GraFit software (Erithacus Software Limited, Middlesex, UK). Shown are representative results of three to five determinations.

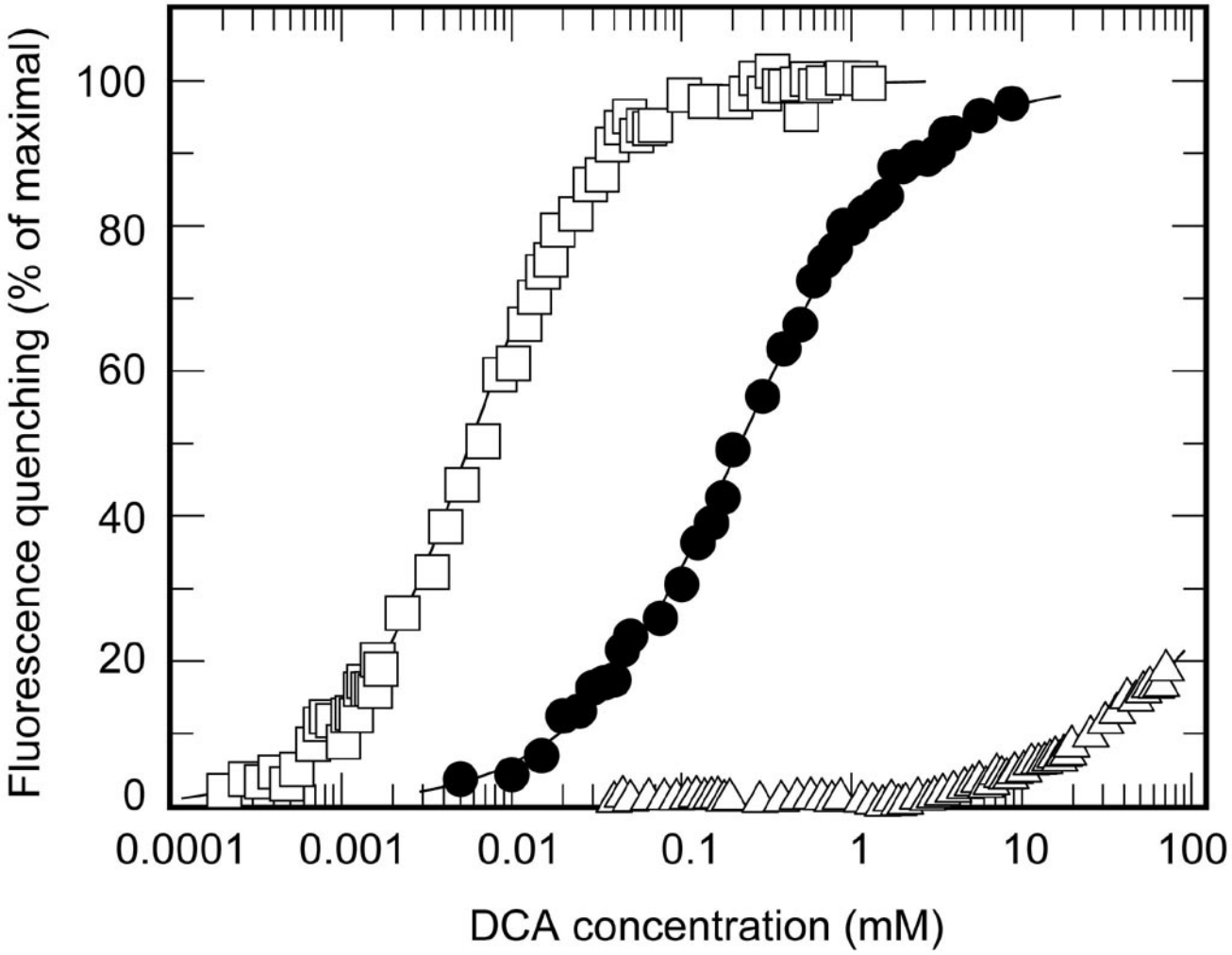

Figure 4. Quenching of the intrinsic PDHK2 fluorescence by DCA

Incremental increases in fluorescence quenching with increasing DCA concentration for wild-type PDHK2, PDHK2-I157A, and PDHK2-R114A are shown by *closed circles*, by *open squares*, and by *open triangles*, respectively. Fluorescence measurements were made as described under "Materials and methods". Fluorescence quenching data were transformed and analyzed using approaches described by Hiromasa and colleagues [12]. Shown are representative results of three to five determinations.

Table 1

Kinetic parameters for inhibition of enzymatic activities of wild-type and mutant PDHK2 proteins by DCA.

PDHK2	IC_{50} for DCA (mM)	Slope factor
wild-type	2.3 ± 0.3,[1,2]	0.6 ± 0.1
L53A	17.1 ± 1.1	0.6 ± 0.2
Y80A	NM[3]	–
S83A	NM	–
I111A	17.9 ± 1.1	0.6 ± 0.1
R112A	1.2 ± 0.4	0.7 ± 0.2
R114A	NM	–
H115A	8.4 ± 0.4	0.8 ± 0.1
D117A	NM	–
R154A	1.7 ± 0.2	0.9 ± 0.1
I157A	0.013 ± 0.002	0.9 ± 0.1
R158A	2.0 ± 0.4	1.0 ± 0.2
I161A	2.7 ± 1.5	0.6 ± 0.2

[1] Kinetic parameters represent the means ± S.D. obtained for three to five independent determinations.

[2] Kinase activity was determined by measuring the initial rate of incorporation of ^{32}P from [γ-^{32}P]ATP into E1α subunit of recombinant E1 as described elsewhere [4]. Prior to the assay, E1, E2, and PDHK2 were reconstituted on ice. The final protein concentrations of the recombinant proteins in the assay were as follows: E1 at 5μM; E2 at 20 nM; and PDHK2 at 20 nM. The results were corrected for non-specific inhibition determined in the presence of increasing concentration of acetate. Background-corrected kinetic data were fitted and analyzed using GraFit software (Erithacus Software Limited, Middlesex, UK).

[3] NM, not measurable.

Table 2

Parameters for DCA binding by the wild-type and mutant PDHK2 proteins.

PDHK2	EC_{50} for DCA (mM)	Slope factor
Wild-type	0.42 ± 0.02[1]	0.79 ± 0.02
L53A	2.09 ± 0.11	0.96 ± 0.05
Y80A	1.98 ± 0.13	0.71 ± 0.03
S83A	NM[2]	–
I111A	1.09 ± 0.07	0.81 ± 0.04
R112A	0.31 ± 0.01	0.95 ± 0.02
R114A	NM	–
H115A	1.46 ± 0.04	1.21 ± 0.03
D117A	0.63 ± 0.02	0.77 ± 0.02
R154A	0.195 ± 0.02	0.98 ± 0.03
I157A	0.0026 ± 0.001	1.02 ± 0.06
R158A	0.12 ± 0.01	1.18 ± 0.04
I161A	0.11 ± 0.02	1.3 ± 0.1

[1] Binding parameters represent the means ± S.D. obtained for three to five determinations.

[2] NM, not measurable.