Evidence that pyruvate dehydrogenase kinase belongs to the ATPase/kinase superfamily

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In this study the roles of invariant Asn-247, Asp-282, Gly-284, Gly-286 and Gly-319 of pyruvate dehydrogenase kinase were investigated by site-directed mutagenesis. Recombinant kinases, wild-type, Asn-247Ala, Asp-282Ala, Gly-284Ala, Gly-286Ala and Gly-319Ala, were expressed in bacteria, purified, and characterized. Three mutant kinases, Asn-247Ala, Asp-282Ala and Gly-286Ala, lacked any appreciable activity. Two other mutants, Gly-284Ala and Gly-319Ala, were catalytically active, with apparent $V_{\max}$ values close to that of the wild-type kinase (67 and 85 versus 70 nmol/min per mg, respectively). The apparent K_m value of Gly-319Ala for nucleotide substrate increased significantly (1500 versus 16 μ M). In contrast, Gly-284Ala had only a slightly higher K_m value than the wild-type enzyme (28 versus 16 μ M). ATP-binding analysis showed that Asn-247Ala, Asp-282Ala and Gly-286Ala could not bind nucleotide. The K_d value of Gly-284Ala was slightly higher than that of the wild-type enzyme (7 versus 4 μ M, respectively). In agreement with kinetic

analysis, the Gly-319Ala mutant bound ATP so poorly that it was difficult to determine the binding constant. Despite the fact that Asn-247Ala, Asp-282Ala and Gly-286Ala lacked enzymic activity, they were still capable of binding the protein substrate, as shown by their negative-dominant effect in the competition assay with the wild-type kinase. The results of CD spectropolarimetry indicated that there were no major changes in the secondary structures of Asp-282Ala and Gly-286Ala. These results suggest strongly that the catalytic domain of pyruvate dehydrogenase kinase is located at the C-terminus. Furthermore, the catalytic domain is likely to be folded similarly to the catalytic domains of the members of ATPase/kinase superfamily [molecular chaperone heat-shock protein 90 (Hsp90), DNA gyrase B and histidine protein kinases].

Key words: catalysis, nucleotide-binding site, protein kinase, protein phosphorylation, site-directed mutagenesis.

INTRODUCTION

The mammalian pyruvate dehydrogenase complex (PDC) is one of the central enzymes of general metabolism that largely defines the metabolic fate of carbohydrate fuels. It catalyses the irreversible decarboxylation of pyruvic acid and, therefore, commits all metabolically related three-carbon compounds derived from carbohydrates to further conversion. Mammalian PDC consists of three enzymes working in a highly co-ordinated manner: pyruvate dehydrogenase or E1 (thiamin pyrophosphate-dependent enzyme); dihydrolipoamide transacetylase or E2 (lipoic acid-dependent enzyme); and dihydrolipoamide dehydrogenase or E3 (flavin-adenine dinucleotide-dependent enzyme) [1,2]. Co-ordination between the different enzymes is achieved through their physical organization in a complex of high molecular mass. This allows for the direct transfer of intermediates from one active site to another. The core of the complex is built of E2 components, organized as a pentagonal dodecahedron. Other enzymes are attached to this core, with E1 components (a tetramer of two E1 α and two E1 β subunits) being positioned on the edges and E3 components (a dimer of identical subunits) being positioned on the faces of the E2 core. The coupling of the active sites is carried out by lipoic acid attached to the lipoyl-bearing domains of the E2 component [1,2].

The enzymic activity of PDC is highly regulated. In animals, it is achieved primarily via the phosphorylation/dephosphorylation cycle [3,4]. The inactivation of PDC occurs via phosphorylation catalysed by a dedicated protein kinase

(pyruvate dehydrogenase kinase or PDH kinase). The kinase is tightly associated with the complex core and phosphorylates three serine residues of the decarboxylase component (E1 α) [4] that leads to inactivation of the holo-complex [5,6]. The phosphoenzyme re-activation requires dephosphorylation catalysed by dedicated pyruvate dehydrogenase phosphatase which, in contrast to the kinase, is loosely associated with PDC [3]. Both the kinase and phosphatase can integrate a considerable number of different regulatory stimuli (reviewed in [7]). In general, the kinase activity reflects the relative concentrations of the substrates (inhibition) and products (activation) of the main dehydrogenase reaction. The enzymic activity of phosphatase depends on the intramitochondrial concentrations of Mg^{2+} , Ca^{2+} , NADH and insulin in several lipogenic tissues [8]. This short-term regulation adjusts flux through PDC on a minute-to-minute basis. Besides, there is also so-called long-term regulation [7]. This term refers to the hyperphosphorylated state of PDC that develops under conditions such as starvation, diabetes, ischaemic heart disease, sepsis and lactic acidosis [7]. In contrast to the short-term effects, long-term effects correlate with the stable increase in the activity of PDH kinase. Recent evidence presented by this laboratory suggests strongly that short-term and long-term regulation of PDC activity may be carried out by different isozymes of PDH kinase [9,10]. To date, four genes have been identified in the human genome that encode the different isozymes of PDH kinase (PDK1, PDK2, PDK3 and PDK4). The contributions of isozymes of PDH kinase to the regulation of PDC activity have not been fully elucidated. It appears that ubiquitously expressed

Abbreviations used: PDH kinase, pyruvate dehydrogenase kinase; PDK1–4, isozymes of PDH kinase; PDC, pyruvate dehydrogenase complex; E1, pyruvate decarboxylase component of PDC; E2, dihydrolipoamide transacetylase component of PDC; E3, dihydrolipoamide dehydrogenase component of PDC; BCODC, branched-chain 2-oxoacid dehydrogenase complex; Hsp90, heat-shock protein 90; EnvZ, bacterial histidine protein kinase involved in osmosensing; CheA, bacterial histidine protein kinase involved in regulation of chemotaxis.

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isozyme PDK2 is likely to contribute the most to short-term regulation [10], whereas the long-term regulation is largely carried out by the inducible isozyme PDK4 [11].

Currently, further progress in studies on PDH kinase is tempered by the lack of understanding as to how this important enzyme functions. By specificity, PDH kinase is a Ser-specific protein kinase [3]; however, its sequence does not show any similarity with the rest of eukaryotic Ser/Thr-specific protein kinases [12,13]. This prompted us to suggest that the catalytic domain of PDH kinase assumes a different folding pattern [12]. The present study was undertaken to test this hypothesis. Here, we report the first evidence suggesting that, indeed, the catalytic domain of PDH kinase is completely different from the catalytic domains of other Ser/Thr-specific protein kinases. It is likely to be folded similarly to the catalytic domains of the members of the ATPase/kinase superfamily [14–17].

EXPERIMENTAL

Modelling of the three-dimensional structure of the putative catalytic domain of PDH kinase

The structural alignment of the protein sequence of rat PDH kinase (isozyme PDK2) with protein sequences of the members of the ATPase/kinase superfamily was generated using the Clustal W program, version 1.7 [18]. The template sequences of histidine protein kinases were from following sources: EnvZ (a bacterial histidine protein kinase involved in osmosensing) from *Escherichia coli* (GenBank accession no. P02933); RstA from *E. coli* (AAC74681); CpxA from *E. coli* (S40855); CheA (a bacterial histidine protein kinase involved in regulation of chemotaxis) from *Thermotoga maritima* (Q56312); PhoR from *Vibrio cholerae* (AAC74681); and BaeS from *E. coli* (P30847). The template sequences of DNA gyrases were from following sources: escGyr from *E. coli* (GenBank accession no. P06982); salGyr from *Salmonella typhimurium* (CAA69665); haeGyr from *Haemophilus influenzae* (AAC22225); pseGyr from *Pseudomonas putida* (P13364); neiGyr from *Neisseria gonorrhoeae* (AAA88327); and bucGyr from *Buchnera aphidicola* (AAC38108).

The template sequences of molecular chaperone heat-shock protein 90 (Hsp90) were from the following sources: danHsp90 from *Danio rerio* (GenBank accession no. AAC21566); humHsp90 from *Homo sapiens* (AAA36026); galHsp90 from *Gallus gallus* (CAA30251); droHsp90 from *Drosophila melanogaster* (2144913); dicHsp90 from *Dictyostelium discoideum* (AAA69917); tryHsp90 from *Trypanosoma cruzi* (AAA30202); leiHsp90 from *Leishmania donovani infantum* (S57415); schHsp90 from *Schizosaccharomyces pombe* (fP41887); and sacHsp90 from *Saccharomyces cerevisiae* (CAA89919).

In order to increase the sensitivity of the alignment, the following sequences of members of the PDH kinase gene family were included in the alignment: humBCODK, the kinase that phosphorylates the branched-chain 2-oxoacid dehydrogenase complex (BCODC) from *H. sapiens* (GenBank accession no. O14874); ratBCODK from *Rattus norvegicus* (AAB60498); musBCODK from *Mus musculus* (AAB97689); zeaPDK1 and zeaPDK2 from *Zea mays* (AAC63961 and ACC63962, respectively); araPDK from *Arabidopsis thaliana* (ACC97601); humPDK1–4 from *H. sapiens* (Q15118, Q15119, Q15120 and AAC50670, respectively); ratPDK2 and ratPDK4 from *R. norvegicus* (Q64536 and Q63065, respectively); musPDK4 from *M. musculus* (CAA04752); spePDK4 from *Spermophilus tri-decemlineatus* (AAC40161); caePDK, PDH kinase from *Caenorhabditis elegans* (Q02332); ascPDK from *Ascaris suum* (O02623); droPDK from *D. melanogaster* (P91622); and sacPDK2 from *S. cerevisiae* (S50696).

The three-dimensional structure of the sequence-aligned rat PDK2 was generated using the MODELLER 4.0 program [19]. The spatial restraints for the modelling were from the three-dimensional structures of DNA gyrase B or molecular chaperones Hsp90 (PDB accession no.s 1AJ6 and 1AM1, respectively). The docking of ADP into the hypothetical active site of PDK2 was done manually using Swiss-PdbViewer program, version 3.1 [20]. Nucleotide (ADP) was positioned in a way it was found in the three-dimensional structure of molecular chaperone Hsp90 (PDB accession no. 1AM1).

Site-directed mutagenesis

Point mutations within the amino acid sequence of rat PDK2 were introduced using oligonucleotide-directed mutagenesis [21]. The sequences of mutagenic oligonucleotides were as follows: 5'-CCG CAT GGC AGC CTT GAA GAG TTC-3' for the Asn-247Ala mutant; 5'-C ACC CCC GCC TCG GGC ACT CAT TTT GAT GG-3' for Asp-282Ala; 5'-GGG GAC ACC CCC TGC TCG GTC ACT CAT TTT GAT GG-3' for Gly-284Ala; 5'-CCT CAA GGG GAC AGC CCC GCC TCG GTC ACT C-3' for Gly-286Ala; and 5'-GGG GAG TCC ATA CGC AAA GCC AGC G-3' for Gly-319Ala (the amino acid residues are numbered according to the sequence of mature rat PDK2; the altered bases are underlined). Mutagenesis reactions were carried out on single-stranded DNA of rat PDK2 [12] subcloned into M13mp19 using the Sculptor[™] site-directed mutagenesis kit (Amersham). The reactions were set up essentially as recommended by the manufacturer. The mutations as well as the fidelity of the rest of DNAs were confirmed by direct sequencing [22].

Expression and purification of the mutant kinases

PDK2 cDNAs (≈ 1.2 kb) carrying the point mutations were cut out of the M13 DNA with *SacI* and *HindIII* restriction endonucleases, and re-ligated into the pET-28a expression vector (Novagen) between the *SacI* and *HindIII* sites of the vector (pPDK2 vector). The transcripts produced from this construct contained the entire coding region of PDK2 along with several vector-encoded sequences for peptide tags located at the 5'-end of the transcript. The resulting kinases, therefore, had a stretch of six consecutive histidine residues (a His₆ tag) at the far N-terminus followed by a short polypeptide allowing the detection of target protein by Western blotting (T7-Tag). Plasmids containing the inserts of the correct size (≈ 1.2 kb) were identified by restriction analysis. Positive plasmids were co-transfected into BL21(DE3) cells (Novagen) with pGroESL plasmid carrying the genes coding for molecular chaperones GroEL and GroES under the control of isopropyl β -D-thiogalactoside-inducible promoter (the respective plasmid was obtained as a generous gift from Dr. Anthony Gatenby at DuPont Central Research and Development, Wilmington, DE, U.S.A.). Double transformants were selected on yeast-tryptone agar containing kanamycin and chloramphenicol (50 μ g/ml each) [10]. Several individual colonies from every transformation were tested for their ability to produce significant amounts of soluble, recombinant kinase using Western blotting with monoclonal antibodies against T7-Tag (Novagen). Clones expressing the greatest amount of the soluble kinase were used for further analysis.

The expression of the mutant kinases was performed essentially as described previously [8]. Their purification was carried out using TALON[™] (Clontech) affinity resin as described elsewhere [10]. The protein composition of each preparation was evaluated by SDS/PAGE analysis. Gels were stained with Coomassie

R250. The purity of all preparations used in this study was greater than 90%.

Kinase activity assay

Kinase activities of the wild-type and mutant PDK2s were determined following the incorporation of [32 P]phosphate into the kinase-free porcine heart PDC from [γ - 32 P]ATP essentially as described previously [10]. For each assay, the kinase was added to the complex at a ratio of two kinase molecules per complex. Under these conditions the phosphorylation reactions are linear in time and the rate of the reaction is proportional to the amount of kinase added. Catalytically inactive mutants (Asn-247Ala, Asp-282Ala, and Gly-286Ala) were also tested in concentrations exceeding 10–15-fold the concentration of kinase in the standard assay. Prior to the initiation of the phosphorylation reaction with [γ - 32 P]ATP (specific radioactivity 200–500 c.p.m./pmol), the samples were incubated at 37 °C for 30 s in order to allow the reaction mixtures to equilibrate. The reactions were terminated after 60 s of incubation. All experiments were performed with negative (minus kinase) and with positive (wild-type kinase) controls. The amount of radioactive [32 P]phosphate incorporated into PDC was determined as described previously [10].

For determination of the kinetic constants, the concentration of ATP used in the reaction mixtures was varied from 5 to 200 μ M for wild-type PDK2 and Gly-284Ala mutant, and from 0.5 to 10 mM for Gly-319Ala mutant. Catalytically inactive mutants (Asn-247Ala, Asp-282Ala, and Gly-286Ala) were tested over a wide range of ATP concentrations from 10 μ M to 10 mM. For determination of the apparent K_i values for ADP, substrate cocktails of ADP and ATP were prepared with five different concentrations of ATP (as described above) and five different concentrations of ADP. The concentration of ADP was varied from 0 to 0.3 mM for wild-type PDK2, from 0 to 2.4 mM for Gly-284Ala and from 0 to 10 mM for Gly-319Ala. This produced a matrix of 25 conditions. The substrate and inhibitor were added simultaneously to the reactions, and samples were treated as described previously [10]. Under conditions in which high concentrations of nucleotides were used, all reactions including controls were set up at a fixed Mg^{2+} concentration of 20 mM. The experiments were conducted in duplicates. Raw kinetics data were fitted and analysed using GraFit software (Erithacus Software) for at least three independent data sets.

Three catalytically inactive mutants of PDK2, Asn-247Ala, Asp-282Ala and Gly-286Ala, were used in a competition assay. For these experiments, the fixed amount of wild-type enzyme was combined with various amounts of appropriate mutant protein. The respective mixtures were used in the standard kinase assay as described above. Determinations were done at a fixed ATP concentration (100 μ M).

ATP-binding assay

Nucleotide-binding studies were conducted using the vacuum-filtration assay developed by Pratt and Roche [23], with some modifications. Briefly, the recombinant kinases in binding buffer (20 mM Tris/HCl, pH 7.4/5 mM $MgCl_2$ /50 mM KCl/5 mM dithiothreitol) were used in a final concentration of 0.1 mg/ml. Binding reactions (total volume 100 μ l) were initiated by the addition of [α - 32 P]ATP (specific radioactivity 200–500 c.p.m./pmol). The final concentration of ATP in the binding buffer was varied from 2.5 to 100 μ M. Reactions were incubated at room temperature for 90 s. At the end of the incubation, binding reactions were terminated by applying to nitrocellulose filters with a pore diameter of 0.2 μ m (Schleicher and Schuell) mounted in the filtration unit (Millipore) under vacuum. Filters were washed

three times, with 3 ml of ice-cold binding buffer per wash, and dried. Bound radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of a 1000-fold excess of unlabelled ATP. Non-specific binding never exceeded 0.5% of the total binding. The values for specific binding were calculated after subtraction for non-specific binding. In preliminary experiments (results not shown) it was established that, under the described conditions, the binding of ATP to PDK2 reaches equilibrium within 60 s of the start of incubation. The binding was readily reversible upon addition of the excess of unlabelled ATP to the binding mixtures. All binding experiments were conducted in triplicates. Binding curves were fitted and analysed using GraFit software for at least three independent data sets.

Other procedures

The CD spectra of wild-type PDK2 and mutant kinases were recorded in 50 mM potassium phosphate, pH 7.5, with 5 mM β -mercaptoethanol (protein concentrations 2–8 μ M) using a Jasco J-720 spectrometer. Secondary structure was calculated using the SSE-338 program and the reference spectra library of Yang et al. [24]. SDS/PAGE was performed according to Laemmli [25]. Western-blot analysis was carried out as described previously [11] with mouse monoclonal antibodies against T7-Tag (Novagen). Antibodies were used at a working dilution of 1:5000. Immunoreactive proteins were visualized using goat anti-mouse IgG antibodies conjugated with peroxidase (Bio-Rad). Blots were stained with 4-chloro-1-naphthol (Sigma). Protein was determined according to Lowry et al. [26] using BSA as a standard.

RESULTS AND DISCUSSION

Molecular modelling of the putative catalytic domain of PDH kinase

Previous analysis of the deduced amino acid sequences of mitochondrial protein kinases has revealed a highly conserved core sequence of $\approx$ 130–150 amino acids located at the C-terminus of the kinase molecule [9]. It is defined by the consensus sequences EXXKNXXA (subdomain II), DXGXG (subdomain III) and GXGXG (subdomain IV) [9]. A similar subdomain arrangement is found in the catalytic domains of several groups of proteins, namely bacterial histidine protein kinases, DNA gyrases and molecular chaperone Hsp90 (the so-called ATPase/kinase superfamily) [14–17]. Despite the low sequence similarity between the latter proteins, their catalytic domains are folded into virtually identical α/β sandwiches [14–17]. This observation suggests that the catalytic domain of PDH kinase might assume a similar fold. To test this intriguing possibility, we used a combination of molecular modelling and site-directed mutagenesis.

To generate the structural alignment for the molecular modelling, the amino acid sequences of mitochondrial protein kinases were aligned with amino acid sequences of histidine protein kinases, DNA gyrases and molecular chaperone Hsp90 (Figure 1). The alignment was generated using Clustal W [18]. The sensitivity of the alignment was improved by using several template sequences for histidine kinases, DNA gyrases and molecular chaperone Hsp90 and by restricting the length of the alignment to the length of the C-terminus of PDH kinase. This approach allowed us to generate a representative alignment. This is shown by the proper positioning of the elements of secondary structure of the template sequences (Figure 1, bottom panel). The analysis also revealed that some basic elements of the secondary structure (helices A1–A3 and strands B1–B3) are

Figure 1 Alignment of the deduced protein sequences of mitochondrial protein kinases and members of ATPase/kinase superfamily (histidine protein kinases, DNA gyrases and chaperone Hsp90)

Sequences were aligned using Clustal W, version 1.7 [18]. The sources of the sequences and their GenBank accession numbers are given in the Experimental section. Hyphens represent gaps that have been inserted to align conserved regions. The amino acid residues of subdomains II, III and IV that are uniformly conserved in all proteins are shown by white text on black boxes. Highly conserved amino acids are indicated under the alignment by colons (:); less-conserved amino acids are indicated by dots (.). The positions corresponding to Glu-42 and His-38 of DNA gyrase B that were implicated in the catalysis [28] are indicated under the alignment by carets (^). The elements of the secondary structures of CheA [15], EnvZ [14], Hsp90 [16] and DNA gyrase [17] are shown at the bottom by arrows for β -strands and by bars for α -helices. The elements of secondary structure that are uniformly conserved among all ATPases/kinases (helices A1–A3, strands B1–B3) are shown underneath. Helices that are unique for CheA and Hsp90 (a1 α , a2 α and a2 β) are shown as grey bars.

Figure 2 A ribbon representation indicating the secondary structural elements in a vicinity of the putative catalytic domain of PDH kinase

The model of the putative catalytic domain of rat PDK2 was built using MODELLER 4.0 [19]. The spatial restraints for the modelling of sequence-aligned PDK2 were from the three-dimensional structure of DNA gyrase B [17] (PDB accession no. 1AJ6). Only those regions of PDK2 that were aligned to the target sequence (DNA gyrase) were modelled. Docking of ADP in the putative active site was done manually using Swiss-PdbViewer [20]. The model is viewed down the edge of the α/β -sandwich structure. The residues of subdomains II, III and IV (Asn-247, Asp-282, Gly-284, Gly-286 and Gly-319, respectively) targeted in this study are shown as ball-and-stick diagrams. The drawing was made using MOLSCRIPT [29].

uniformly conserved among all ATPases/kinases. Other structural elements (helices $\alpha 1$ in CheA, $\alpha 2$ in Hsp90 and CheA, and helix $\alpha 2\beta$ in Hsp90), in contrast, are unique, reflecting the difference in the length of spacers connecting the subdomains II, III and IV, characteristic of CheA and Hsp90 [14,15]. In general, the alignment correctly predicted the elements of the secondary structure of ATPases/kinases, and therefore was used to model the putative catalytic domain of PDH kinase. The three-dimensional structure of the catalytic domain of PDH kinase (rat isozyme PDK2) was built using the program MODELLER 4.0, which models three-dimensional structure by satisfying spatial restraints [19]. The input restraints used were from the crystal structure of DNA gyrase B or molecular chaperone Hsp90. Only those regions that could be aligned to the target sequence (DNA gyrase B or chaperone Hsp90) were modelled. The output of the program (Figure 2) suggested that the amino acid sequence of rat PDK2 could be folded analogously to DNA gyrase or molecular chaperone Hsp90 (results not shown) and, probably, to other ATPases/kinases (molecular co-ordinates for histidine protein kinases CheA and EnvZ are currently unavailable) without any conformational difficulties. The latter suggests that the key residues defining the subdomains II, III and IV of PDK2 (Asn-247 of subdomain II, Asp-282, Gly-284 and Gly-286 of subdomain III, and Gly-319 of subdomain IV) might be equivalent to the respective residues of ATPases/kinases, which are intimately involved in anchoring of the nucleotide substrate by donating their side chains for binding (Asn and Asp residues) or by allowing the main chain to contour the nucleotide (Gly residues) [14–16].

Site-directed mutagenesis and expression of the mutant kinases

In order to test the hypothesis that subdomains II, III and IV of PDH kinase contribute to the nucleotide-binding site, we used

alanine-scanning mutagenesis targeting the consensus-defining amino acids: Asn-247, Asp-282, Gly-284, Gly-286 and Gly-319. Point mutations were introduced using oligonucleotide-directed mutagenesis. The cDNAs carrying the mutations were inserted into bacterial expression vector, pET-28a, and expressed in *E. coli* strain BL21(DE3), as described in the Experimental section. Mutant kinases were purified using affinity chromatography on TALON[®] resin. SDS/PAGE analysis of the respective protein products revealed that all mutant kinases were more than 90% pure (results not shown). Four out of five mutants were expressed at levels similar to the wild-type enzyme. For unknown reasons, the level of expression of the Asn-247Ala mutant was substantially lower than that of the others. In order to evaluate the impact of the mutations on the enzymic properties of PDK2, the mutant proteins along with wild-type enzyme were subjected to several functional and structural assays: activity assay, ATP-binding assay, competition assay and CD spectroscopy. The low level of expression of the Asn-247Ala mutant prevented its analysis using procedures requiring relatively high amounts of protein, such as CD spectroscopy.

Analysis of the mutant enzymes

Initially, all mutant kinases were analysed for their ability to phosphorylate PDC. As shown in Table 1, mutant kinases Asn-247Ala and Asp-282Ala were found to be catalytically inactive. This appeared to be the case even when their activities were tested using protein concentrations exceeding the concentration of the wild-type enzyme by 10–15-fold, with ATP concentrations as high as 10 mM (the apparent K_m value for the wild-type PDK2 for ATP was $\approx 16 \mu\text{M}$). A similar analysis for glycine mutants, Gly-284Ala and Gly-286Ala (subdomain III) and Gly-319Ala (subdomain IV), revealed that their capacity for catalysis varied widely. The mutant Gly-284Ala PDK2 was completely

Table 1 Kinetic parameters of the wild-type and mutant enzymes

Kinetic parameters were determined as described in the Experimental section using the [32 P]phosphate-incorporation assay. Values are presented as means $\pm$ S.E.M. for $n = 3$ –5 independent enzyme preparations. NA, not active.

Enzyme	$V_{\max}$ (nmol/min per mg)	Apparent K_m for ATP (μ M)	Apparent K_i for ADP (μ M)
Wild-type PDK2	70 ± 2	16 ± 1	200 ± 20
Asn-247Ala PDK2	NA	—	—
Asp-282Ala PDK2	NA	—	—
Gly-284Ala PDK2	67 ± 2	28 ± 4	300 ± 30
Gly-286Ala PDK2	NA	—	—
Gly-319Ala PDK2	85 ± 5	1500 ± 300	1600 ± 100

active, showing just a slight increase in the apparent K_m value for ATP (28 versus 16μ M for the wild-type PDK2) and in the apparent K_i value for ADP (300 versus 200μ M for the wild-type PDK2). In contrast, substitution of the next conserved glycine residue in subdomain III (Gly-286Ala) resulted in completely inactive kinase. No activity was detected, even when using a high amount of this mutant kinase with high concentrations of ATP in the assay. Gly-319Ala was the most intriguing of all. Its $V_{\max}$ value was slightly higher than that of the wild-type enzyme (85 versus 70 nmol/min for the wild-type PDK2; Table 1). However, its catalytic efficiency ($V_{\max}/K_m$) was more than 75-fold lower. This effect was due largely to the dramatic increase in the apparent K_m value for ATP (1500 versus 16μ M for wild-type PDK2). The apparent K_i value for ADP increased 8-fold (Table 1), suggesting that the Gly-318Ala mutation allowed discrimination between binding of ATP and ADP in the active site.

In order to test the possibility that the catalytically inactive mutants might have had grossly altered conformations, we evaluated the secondary structures of Asp-282Ala and Gly-286Ala using CD spectroscopy. Calculation of the secondary structures based on CD spectra revealed that $\approx 40\%$ of the sequence of mutant kinases had α -helical conformation and $\approx 30\%$ of the sequence was in combined β -conformation. These values are very close to those for the wild-type enzyme (41 and 29% , respectively). This outcome suggests that the effect of these mutations is due to interference with some steps in binding of the nucleotide or protein substrates and/or catalysis, rather than because of interference with protein folding.

To test this idea further, we analysed mutant kinases for their ability to bind nucleotide (ATP) and protein (PDC) substrates. Nucleotide binding was assayed using a vacuum-filtration technique with [α - 32 P]ATP as a ligand. In accord with the results of the activity assay, the catalytically inactive mutants (Asn-247Ala, Asp-282Ala and Gly-286Ala) did not show significant binding over the background values (results not shown). In contrast, Gly-284Ala and Gly-319Ala mutants were found to bind [α - 32 P]ATP (Figure 3). The K_d value for Gly-284Ala was slightly higher than that of the wild-type enzyme (7 versus 4μ M, respectively). In contrast, Gly-319Ala bound ATP so poorly that it was difficult to determine the binding constant. However, under all concentrations of ATP tested, the values for specific binding always exceeded the values for non-specific binding. This outcome is in agreement with the results of the kinetic analysis showing that the apparent K_m value for ATP for Gly-319Ala was almost 100-fold higher than that of the wild-type enzyme (Table 1).

A competition assay was used to test whether catalytically inactive mutants Asn-247Ala, Asp-282Ala and Gly-286Ala were

Figure 3 [α - 32 P]ATP binding by the wild-type PDK2 and Gly-284Ala and Gly-319Ala

Recombinant wild-type PDK2 (●) and the Gly-284Ala (■) and Gly-319Ala (□) mutants were incubated with the indicated concentrations of [α - 32 P]ATP (specific radioactivity 200–500 c.p.m./pmol) for 90 s. Free and protein-bound nucleotides were separated using vacuum-filtration, as described in the Experimental section. The specific binding was determined after subtraction of non-specific binding from the total binding. The non-specific binding was determined in the presence of a 1000-fold excess of unlabelled ATP. Data were fitted based on the single-binding-site model using GraFit software.

still capable of binding the protein substrate (PDC). As shown in Figure 4, the addition of increasing amounts of Asn-247Ala, Asp-282Ala or Gly-286Ala mutants to a fixed amount of wild-type PDK2 in the standard kinase assay led to the progressive decrease in the activity of wild-type enzyme. This result suggests strongly that Asn-247Ala, Asp-282Ala and Gly-286Ala retained some functionality and could still bind the protein substrate. This observation is consistent with the idea that these mutants

Figure 4 Inhibition of the wild-type PDK2 enzymic activity by Asn-247Ala, Asp-282Ala and Gly-284Ala mutants

The experiments were carried out as described in the Experimental section using the standard kinase assay. Indicated amounts of Asn-247Ala (■), Asp-282Ala (●) and Gly-284Ala (○) were added to the PDC in mixtures with a fixed concentration of wild-type PDK2. Phosphorylation reactions were initiated with [γ - 32 P]ATP at a final concentration of 100μ M.

lack catalytic activity due, primarily, to the severely impaired ability to bind the nucleotide substrate.

The molecular basis for the effects of mutations on kinase activities that were observed in this study becomes quite apparent, if it is assumed that the catalytic domain of PDH kinase folds similarly to the catalytic domains of ATPases/kinases. In ATPases/kinases the invariant asparagine residue binds directly to the β -phosphate of ATP and to the Mg^{2+} and indirectly to the adenine base [14–16]. The aspartate residue interacts with the N-6 atom in the adenine base [14–16]. Accordingly, the respective mutants of PDK2, Asn-247Ala (subdomain II) and Asp-282Ala (subdomain III), were inactive and incapable of binding nucleotide (Figure 2). In ATPases/kinases the glycine residues that follow invariant aspartate in the consensus sequence DXGXXG allow the sharp kinks between strand B2 and helix A2 (Figure 1). The latter, in turn, allows the main chain to contour the adenine ring (Figure 2). Furthermore, the first glycine residue in the consensus DXGXXG appears to be relatively far away from the adenine base. The second glycine, in contrast, is almost in direct contact with the base. As a result, the position corresponding to the first glycine can accept the alanine residue (histidine protein kinase BaeS in Figure 1), whereas the second position is exclusively occupied by a glycine residue (Figure 1). The respective Gly-284Ala (subdomain III) mutant of PDK2 was found to be active. The Gly-286Ala (subdomain III) mutant, in contrast, was inactive and could not bind ATP. Finally, in ATPases/kinases the second glycine residue in the consensus sequence GXGXXG allows the main chain to make a sharp turn in the vicinity of β - and γ -phosphates of the nucleotide substrate [15,16] and to contour the polyphosphate moiety of the nucleotide. This implies that the corresponding alanine mutant (Gly-319Ala) of PDK2 has to have an impaired ability to bind the nucleotide substrate. The additional methyl group of alanine has to interfere with the nucleotide binding because of the spatial problems it creates (Figure 2). The corresponding mutation in PDK2 affected the binding of ATP more severely than that of ADP, suggesting that the main chain of PDK2 may make a turn in the vicinity of γ -phosphate.

Despite the remarkable similarities found in the three-dimensional structures of catalytic domains of different ATPases/kinases, the molecular circuitry involved in the catalysis of phospho-transfer appears to be somewhat different between ATPases and kinases. In ATPases, the catalytic domain itself provides the catalyst for activation of the nucleophile (a water molecule). Studies on DNA gyrase have established that the invariant glutamate residue (Glu-42) serves as general base (indicated in Figure 1 by $\wedge$) [27]. It is polarized and aligned by the neighbouring His-38 [27]. Histidine kinases, in contrast, lack the respective Glu/His relay. Their phospho-accepting histidine residue, which is located at the N-terminus of the kinase molecule, is believed to attack the γ -phosphate of ATP directly [15], although its nucleophilicity might be increased through interaction with a neighbouring glutamate residue (His-47 and Glu-70 couple in CheA) [28]. Thus in histidine protein kinases the N-terminal domain complements the catalytic centre, providing a nucleophile for phospho-transfer and activating glutamate. Interestingly, the glutamate residue corresponding to Glu-42 of DNA gyrase appears to be conserved uniformly in all mitochondrial protein kinases identified to date (Glu-243 of rat PDK2). The latter suggests that the mitochondrial protein kinases might use a general base catalysis mechanistically similar to

ATPases to activate the serine residue of protein substrate. Furthermore, most of PDH kinases also contain a histidine residue in the position corresponding to His-38 of DNA gyrase (His-239 of rat PDK2) which, therefore, might be involved in polarization of Glu-243 (Figure 1). BCODC kinase, in contrast, does not have a histidine residue, even in the vicinity of the respective position. On average, this enzyme has a several-fold lower catalytic efficiency than PDH kinase. The latter is consistent with results of site-directed mutagenesis studies published for DNA gyrase [27]. Undoubtedly, the final analysis and the assignment of the residues can be made only after the three-dimensional structures of mitochondrial protein kinases are determined, but the above analysis suggests strongly that the mitochondrial protein kinases belong to the superfamily of ATPases/kinases.

This work was supported by grant GM 51262 from the U.S. Public Health Services and an American Heart Association, Indiana Affiliate, predoctoral fellowship (to M.B.-K.). The authors would like to thank Dr. Robert Harris for his support, fruitful scientific discussions and critical reading of the manuscript.

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