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Biochimica et Biophysica Acta 1748 (2005) 20–25

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Changing the metal ion selectivity of rabbit muscle enolase by mutagenesis: effects of the G37A and G41A mutations

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Received 21 April 2004; received in revised form 18 October 2004; accepted 30 November 2004
Available online 16 December 2004

Abstract

During the reaction catalyzed by enolase, a mobile loop, residues 36–45, closes over the active site. In order to probe the role of this loop movement in catalysis, the glycines at positions 37 and 41 of rabbit muscle enolase (ββ) have been mutated to alanines. The mutant forms–G37A and G41A–of enolase are both active, but have altered selectivity for divalent cations. G37A, when assayed with Mg^{2+}, has 12% the activity of the wild type. However, it is twice as active as wild type when assayed with Mn^{2+}, Zn^{2+}, or Co^{2+}. G41A has 4% the activity of the wild type with Mg^{2+}, is more active than wild type with Co^{2+}, and slightly less active than wild type with Mn^{2+} and Zn^{2+}. The kinetic isotope effect for both mutants is greater than that of the wild type with all 4 divalent cations. These results indicate that the flexibility of this loop has subtle effects on catalytic activity.
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Keywords: Enolase; Mutagenesis; Divalent cation

1. Introduction

Enolase (EC 4.2.1.11) catalyzes the reversible dehydration of 2-phosphoglyceric acid (PGA) to form phosphoenol pyruvate (PEP). All enolases have an absolute requirement for divalent cations. Two divalent cations bind per active site, with the order of binding being (1) divalent cation, (2) substrate, and (3) divalent cation. The chemical reaction is believed to proceed step-wise [1]. The proton on carbon-2 is removed, forming a carbanion (enolate), followed by the loss of the hydroxyl from carbon-3 and the formation of the product. A variety of experimental approaches have demonstrated that a conformation change in the protein occurs upon binding either substrate or the second divalent cation. A variety of divalent cations can bind to the active site; the highest activity is observed with Mg^{2+}. Most mechanistic studies have been done with Mg^{2+} or Mn^{2+}, using enolase from either yeast or rabbit muscle. Mammals have three genes for enolase; the resulting proteins are called α-, β-, and γ-enolase. The predominant isozyme expressed in skeletal muscle is ββ, a dimer of β subunits.

The X-ray crystal structures of yeast enolase in the presence and absence of substrate or analogues have been determined [2–4]; the structures of the enzyme from lobster [5], *T. brucei* [6], *E. coli* [7], and *Enterococcus hirae* [8] are also available. Most enolases are dimeric, with identical subunits. Each subunit is comprised of two domains. The larger domain is a α/β barrel, with the active site at the bottom of the barrel. The smaller, N-terminal domain (residues 1–142), contains a three-stranded β-sheet. One face of this sheet interacts with the large domain of the other subunit; the other face is covered by several helices. There are three loops that have high mobility in the holoenzyme, as evidenced by the high temperature factors [2,5,9]. A comparison of the structures of the holo-enzyme and the enzyme plus substrates or analogues shows that these three loops occupy different positions in E and ES. The three

Abbreviations: PGA, 2-phosphoglyceric acid; PEP, phosphoenolpyruvate; PEG, polyethylene glycol; CD, circular dichroism; k.i.e., kinetic isotope effect

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doi:10.1016/j.bbapap.2004.11.020

loops consist of residues 36–45, 153–169, and 250–267. Loop 1, residues 36–45, closes over the active site, with serine 39 providing two ligands, the carbonyl and the side chain-OH, to the second divalent cation. The other ligands to this cation are two water molecules and two substrate oxygens. The movement of loop 2, residues 153–169, brings His 159 close enough to interact with the substrate. The third loop (250–267) does not contribute directly to the active site, although its movement appears to be linked to that of the 153–169 loop. The interaction of serine 39 with the divalent cation appears to be necessary for catalysis. Two groups have mutated this residue, in the yeast enzyme, to alanine; the k_{cat} of this enzyme, when assayed with Mg^{2+}, is about 0.01% that of the wild-type enzyme [10,11].

In previous work [12], I have studied the mechanism of rabbit muscle enolase, using Mn^{2+} as the cation. At pH 7.1, the slow steps in the reaction are the dissociation of the product and a conformational change. The loop that closes over the active site has glycine residues at positions 37 and 41. I have changed these glycines to alanine in the hopes of perturbing either the rate of loop opening and closing and/or the precise conformation of the active site of the enzyme. The kinetic properties of the resulting mutant forms of enolase–G37A and G41A–show that metal ion selectivity is the result of a delicate balance of factors.

2. Materials and methods

Wild-type rabbit muscle enolase was purchased from Boehringer Mannheim. Polyethylene glycol (PEG)1000 was from Fluka; chelex resin was from Bio-Rad. PGA was purchased from both Boehringer Mannheim and Sigma. [2-^{2}H]2-phosphoglycerate was prepared enzymatically according to Shen and Westhead [13], with slight modifications [14]. The mutagenic oligopeptides were purchased from BioCorp Inc., Montreal.

Plasmid isolation was performed using an alkaline lysis/PEG method [15]. Mutagenesis was performed using the Quick Change (Stratagene) method. The primer sequences were 5′-cga ttc cga gca gc**t** gtg ccc agc g**c**a gct tcc acg g (G37A) and 5′-gga gct tcc acg g**c**g atc tat gaa gc**g** ctg gag ctg aga gat gg (G41A). Each sequence differs from the wild type at two positions, shown in bold. A glycine codon was changed to an alanine codon, and a silent mutation was introduced that produced a new restriction site that could be used for screening purposes (Pvu II for G37A, Eco47III for G41A). Sequencing was performed by Bio S&T, Lachine, Quebec. The expression and purification of enolase were as previously described [16].

Enzyme activity was measured at 25 °C by following the conversion of PGA to PEP at 240 nm. During the purification, the assay buffer contained 50 mM imidazole, pH 7.1, 250 mM KCl, 1 mM magnesium acetate, and 0.1 mM EDTA. Protein was measured with the BioRad protein reagent using bovine serum albumin as standard. For kinetic studies, protein was determined by the absorbance at 215 and 225 nm [17].

The assay buffer for kinetics contained 25 mM Mes and 25 mM Tris, pH 7.1. Both the buffer and the PGA solutions were passed through a chelex column and stored in plastic containers in order to decrease the concentration of extraneous divalent cations. The concentrations of these solutions of PGA were determined using the pyruvate kinase, lactate dehydrogenase linked assay.

Activity was measured in the presence of fixed [Me^{2+}] and varying PGA or 1 mM PGA and varying [Me^{2+}]. The latter protocol was used for determining divalent cation specificity and the effects of Li^+ on activity. Viscosity effects were also studied using this protocol, with assay buffer containing 12% PEG 1000, which has a relative viscosity of 2. In all kinetic experiments, assays were performed in duplicate. The sample compartment of the spectrophotometer was thermostated at 25 °C. ENZFITTER (Biosoft) was used to fit the kinetic data to the standard Michaelis–Menton equation, or to either of two equations that included substrate (Me^{2+}) inhibition:

$$v = (V_{max} \times [S])/(K_m + [S] + ([S]^2/K_i)) \quad (1)$$

$$v = (V_{max} \times [S] + V_2[S]^2/K_i)/(K_m + [S] + ([S]^2/K_i)) \quad (2)$$

Eq. (1) is the equation for substrate inhibition in which the binding of an additional molecule of S results in complete inactivation of the enzyme. Eq. (2) is a modified form in which the inhibited enzyme retains partial activity; V_2 is the residual activity. It was not always possible to make a clear distinction between these two kinetic models of inhibition, since, as K_i increases or V_2 decreases, the equations converge. The kinetic constants reported here are from the equation gave the best fit. The extinction coefficient for PEP in the presence of Me^{2+} was measured and used in the calculation of k_{cat}. The values used were 1.376 mM^{-1} cm^{-1} (Mg^{2+}), 1.308 mM^{-1} cm^{-1} (Mn^{2+}), 1.247 mM^{-1} cm^{-1} (Zn^{2+}), and 1.348 mM^{-1} cm^{-1} (Co^{2+}). Standard deviations of the fits were usually ≤ 5% for k_{cat}, and ≤ 10% for K_m and K_i.

For E-Mg and E-Mn, enzyme was dialyzed against buffer containing the desired Me^{2+}; for studies with Zn^{2+} and Co^{2+}, E-Mg or E-Mn was passed through a small (1 ml) column of chelex and either used immediately or converted to E-Zn or E-Co by the addition of the appropriate Me^{2+}.

Circular dichorism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter; the sample compartment was thermostated at 20 °C. Samples were scanned from 320 to 250 nm (aromatic region) or from 260 to 200 nm (peptide bond), at 20 nm/s, 1 nm band width, and a 1 s response time. Four scans were averaged; baseline subtraction and smoothing were done using the Jasco software.

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