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DIHYDROOROTASE FROM CLOSTRIDIUM OROTICUM IS AN ALLOSTERIC ENZYME

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SUMMARY

Dihydroorotase from Clostridium oroticum exhibits allosteric behavior with respect to both of its substrates. L-dihydroorotate dependence reflects a positive homotropic interaction for which the Hill coefficient is 1.3-1.6, depending upon the preparation. Conversely, a negative homotropic response is observed when L-ureidosuccinate serves as substrate, as characterized by a Hill coefficient of 0.65-0.75. Interaction between L-dihydroorotate binding sites is a labile characteristic lost during enzyme purification. Negative cooperativity of ureidosuccinate binding appears to be more stable. The effects of purification and medium are also discussed.

INTRODUCTION

Dihydroorotase, (4,5-L-dihydro-orotate amidohydrolase E.C.3.5.2.3.), catalyzes the reversible ring closure of L-ureidosuccinate to produce L-dihydroorotate; a principal reaction of the pyrimidine biosynthetic pathway. In the anaerobic bacterium, C. oroticum, dihydroorotase, along with several other enzymes of the orotic acid pathway, is induced by growing the organism on orotate enriched media (1). That the induced enzyme is identical to the constitutive enzyme has not been demonstrated. It has been proposed that dihydroorotase may be a potential regulatory site for pyrimidine biosynthesis (2,3). The enzymes from E. coli, rat liver, and ascites hepatomas exhibit feedback inhibition by orotic acid (1,4,5). This constitutes the first report that dihydroorotase from any source is a regulatory enzyme by virtue of possessing allosteric properties.

MATERIALS AND METHODS

All reagents and reaction mixtures were prepared in glass distilled, deionized water. L-dihydroorotate (L-DHOA), D,L-ureidosuccinate (D,L-USA),

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2(N-morpholino)ethane sulfonic acid (MES) and tris(hydroxymethyl)aminomethane (Tris) were from Sigma Chemical Co. Hydroxylapatite and diethylaminoethyl (DEAE) A50 Sephadex were from Bio Rad and Pharmacia, respectively.

C. oroticum were grown on the orotic acid enriched media of Friedman and Vennesland (6) as modified by Sander et al (7). Bacterial cell pastes were sonicated as reported (7) and the enzyme purification scheme described by Taylor et al (8) employed.

Both the ring closure and hydrolytic activities of dihydroorotase were measured taking advantage of dihydroorotate absorption at 230 nm; $\epsilon = 1.17 \times 10^3\ M^{-1}$ cm (7). Reaction mixtures for dihydroorotate synthesis contained 200 µmoles MES-NaOH buffer (pH 6.0), 0.30 µmoles $ZnSO_4$, 25 µmoles D,L-USA and enzyme in a final volume of 3.0 ml. The reaction mixtures for ring hydrolysis contained 300 µmoles Tris-acetate buffer (pH 8.0), 0.30 µmoles $ZnSO_4$, 3.6 µmoles L-DHOA and enzyme in a final volume of 3.0 ml (7,8). In these assays all reaction components, including enzyme, were pre-incubated at 30°C for 10 minutes, followed by initiation with substrate (30°C). When the effect of enzyme storage conditions were being investigated, reactions were initiated with enzyme. Initial velocities were measured by following either L-DHOA formation or hydrolysis, using a Cary 118 C recording spectrophotometer equipped with cell compartments thermostatted at 30.0°C. Under these conditions, initial velocity is directly proportional to enzyme concentration. One unit of dihydroorotase activity is defined as the quantity of enzyme required to catalyze either the synthesis or hydrolysis of 1.0 mµmole L-DHOA per minute at 30°C. Enzymatic activity is expressed as units of enzyme per ml of enzyme solution added to the reaction mixture. Specific activity refers to units of enzyme catalyzing L-DHOA production per mg protein.

Protein concentration was measured using the colorimetric method developed by Bradford (9).

In experiments requiring the alteration of phosphate concentration or ionic strength of the enzyme in either incubation buffers or reaction mixtures, pH was carefully maintained by titration with minute amounts of 0.10 M HCl or NaOH.

RESULTS AND DISCUSSION

L-Dihydroorotate Dependence

Patrially purified dihydroorotase exhibits a positive homotropic response to the substrate L-DHOA, when assayed for hydrolytic activity. Figure 1 shows that a parabolic curve is obtained when the substrate dependence is plotted by the double-reciprocal method. The Hill coefficient (N_H) ranges from 1.3 to 1.6, depending on the particular enzyme preparation. This value is indicative of at least two moderately cooperative L-DHOA sites per enzyme molecule.

Upon further enzyme purification, employing a previously reported method (8), the allosteric character of the enzyme is no longer evident. Substrate (L-DHOA) kinetics become reduced to those of a simple Michaelis-Menton enzyme system, as evidenced by a hyperbolic velocity-substrate relationship ($N_H = 1.0$). The K_m for L-DHOA determined for non-allosteric enzyme prepara-

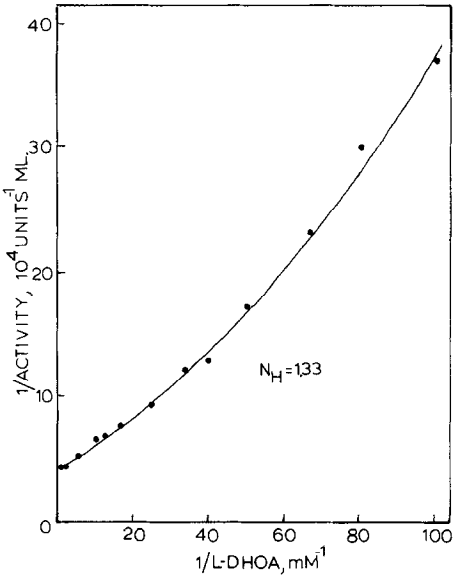


Figure 1

Positive homotropic response of dihydroorotase catalyzed hydrolysis of L-dihydroorotate, 30°C, pH 8.0. Enzyme specific activity was 4240 units mg^{-1} protein. N_H refers to the Hill coefficient of this particular preparation.

tions equals 0.035 mM, a value significantly lower than the previously reported value of 0.07 mM (8). Loss of sigmoidal substrate kinetics has been similarly observed during the purification of E. coli threonine deaminase (10).

Dihydroorotase retains its allosteric response to L-DHOA throughout both DEAE A50 Sephadex steps of the purification scheme. However, following elution from hydroxylapatite, hyperbolic substrate dependence (N_H = 1.0) is observed. Thus, this step of the purification could have removed a regulatory component which elicits an allosteric response from dihydroorotase toward L-DHOA. To test this hypothesis, the protein eluted from the hydroxylapatite column was divided into five major peaks, one of which contained "desensitized" dehydroorotase, i.e. exhibiting hyperbolic kinetics (Figure 2). Each of the four pooled protein fractions was added back, both separately and collectively, to the non-allosteric dihydroorotase fractions. In no case did reconstitution induce cooperative L-DHOA binding.

Since phosphate has been reported to be a highly unstable medium for DHOtase, due to the zinc metallo-nature of the enzyme (7,8), highly purified

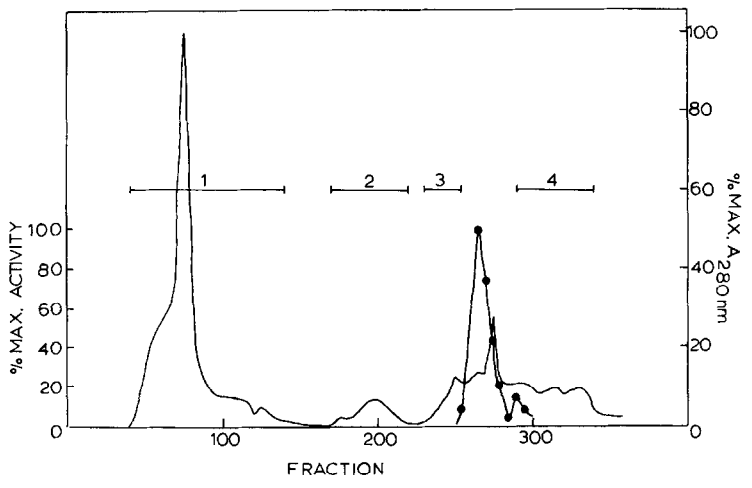
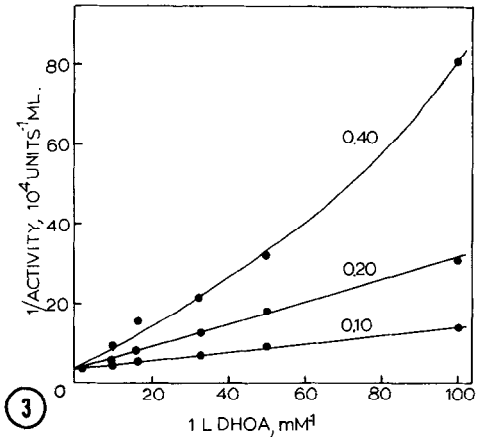


Figure 2

Hydroxylapatite chromatography of allosteric dihydroorotase. 179,500 units of dihydroorotase of specific activity 5679 units mg^{-1} protein were adsorbed on a 3 x 30 cm column. Elution was accomplished with a linear 2 liter gradient of 0.10 to 0.50 M potassium phosphate buffer (pH 6.8). Recovery of dihydroorotase after chromatography was 85%. Numbers and bars represent the fractions which were combined and added back to the dihydroorotase containing fractions.

non-allosteric dihydroorotase was incubated for 3 days at 4.0°C in buffers (pH 7.6) of constant ionic strength (μ = 0.3 M) and varying phosphate concentration (0.01-0.10 M). Under these conditions, phosphate did not induce allosteric kinetics with respect to L-DHOA nor did it result in lowered activity. To further examine medium effects on the allosteric nature of the enzyme, non-allosteric dihydroorotase was incubated for 2 days in 0.01 M Tris-HCl buffers (pH 7.6) of varying ionic strength (0.01-0.40 M) by the addition of NaCl. In this experiment, incubation in buffer of 0.40 M ionic strength induced allosteric kinetics with respect to L-DHOA. To extend these studies, L-DHOA kinetics were examined in standard reaction mixtures (pH 8.0) in which ionic strength was adjusted to 0.10, 0.20 or 0.40 M by NaCl addition. Figure 3 illustrates the resulting Lineweaver-Burk plots. The Hill coefficients obtained at 0.10, 0.20 and 0.40 M ionic strength were 0.90, 1.0 and 1.4, respectively. Both N_H and the $[S]_{0.50}$ (11) increase with increasing NaCl concentration. The kinetics appear to describe a competitive-type inhibition either by sodium or chloride ions in conjunction with the induction of sigmoi-



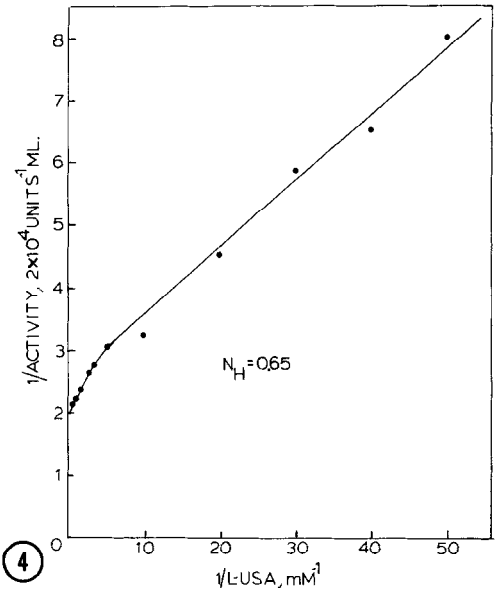


Figure 3

Influence of ionic strength on the cooperativity of dihydroorotase catalyzed L-dihydroorotate hydrolysis, 30°C, pH 8.0. Ionic strength was varied by NaCl addition. Enzyme specific activity was 50,000 units mg^{-1} protein. Numbers refer to the ionic strength of the reaction medium.

Figure 4

Negative cooperativity for the dihydroorotase catalyzed ring closure of L-ureidosuccinate, 30°C, pH 6.0. Enzyme specific activity was 74,000 units mg^{-1} protein. N_H refers to the Hill coefficient of this particular preparation.

dal kinetics. The effect of NaCl, however, does not sufficiently explain the observed allosteric behavior of less purified enzyme preparations, which prior to kinetic analysis were exhaustively dialyzed to remove NaCl.

L-Ureidosuccinate Dependence

The previously reported K_m for L-USA is 0.13 mM (8); in close agreement with the value obtained at high substrate concentrations. However, when a wider substrate range is examined, bi-phasic kinetics are observed on a double-reciprocal plot (Figure 4). Saturation kinetics of this type, which appear to reflect a substrate activation phenomenon, are more aptly viewed as a decrease in the apparent K_m with decreasing substrate concentration. Extrapolation from the high L-USA range on the Lineweaver-Burk plot yields a K_m of 0.094 mM, while extrapolation from the low L-USA range gives a value of 0.049

mM. Hill analysis yields $[S]_{0.50}$ and N_H values of 0.06 mM and 0.75, respectively. The allosteric response to L-USA appears stable to enzyme purification.

Several models can be postulated which fit our data for L-USA dependence. The first requires two discreet catalytic sites with different intrinsic affinities for L-USA. The second involves a negative homotropic interaction between L-USA binding sites. In view of the allosteric response observed for the hydrolytic reaction, it seems likely that a model involving negative cooperativity represents the true kinetic situation.

Ligand induced changes in K_m provides one means of regulating enzymatic activity. We are currently in the process of investigating possible metabolic consequences of the allosteric nature of dihydroorotase. Further characterization of the effects of other ligands on "desensitized" dihydroorotase is currently under investigation.

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