Biochimica et Biophysica Acta, 403 (1975) 232—244

BBA 67608

THE NATURE OF THE MULTIPLE FORMS OF D-*ERYTHRO*-DIHYDRONEOPTERIN TRIPHOSPHATE SYNTHETASE

RAYMOND J. JACKSON and TETSUO SHIOTA

Department of Microbiology, University of Alabama in Birmingham, Birmingham, Al. 35294 (U.S.A.)

(Received April 15th, 1975)

Summary

1. Three forms of the *Lactobacillus plantarum* enzyme D-*erythro*-dihydroneopterin triphosphate synthetase, the first enzyme in folate biosynthesis, have been demonstrated by polyacrylamide gel electrophoresis. The enzyme forms designated the α prime, α and β forms have been shown to be conformers with molecular weights of approx. 200 000. Study of the subunit structure of the β enzyme species by sodium dodecylsulfate-polyacrylamide gel electrophoresis revealed a single protein with an estimated molecular weight of 20 000 which suggests that the enzyme molecule may be composed of ten polypeptide chains.

2. Of the three conformers only one form, the β form, appears to be enzymatically active. The two other conformers must undergo conformational changes to the β species before enzymatic activity can be demonstrated in reaction mixtures containing these enzyme forms.

3. The three enzyme species are interconvertible. The removal of phosphate ions from the enzymatically active β form results in the formation of two inactive species which suggests that the conformation of the active enzyme is stabilized by non-covalently bound phosphate ions. Conversion of the inactive species to the β enzyme form may be effected by the readdition of phosphate, substrate or certain nucleotides.

Introduction

It is now well established that guanosine triphosphate serves as the substrate for an enzyme D-*erythro*-dihydroneopterin triphosphate synthetase [1] (also known as GTP cyclohydrolase [2,3]) which catalyzes the formation of D-*erythro*-dihydroneopterin triphosphate, the initial pteridine precursor for folic acid biosynthesis. In a previous publication [1] we demonstrated that in *Lactobacillus plantarum* this enzyme may exist in at least two enzyme forms which are separable by DEAE-cellulose chromatography. This report describes

some characteristics of these multiple forms in enzyme preparations from *L. plantarum*.

Experimental Procedure

Materials. All chemicals were of reagent grade when possible. DEAE-cellulose was purchased from Schwartz/Mann and prepared for use as described previously [4]. Sepharose 4B and GTP were obtained from the Sigma Chemical Co., ϵ-aminocaproic acid methyl ester from Cyclo Chemical. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Organic Chemicals. [8-^{14}C]GTP (57 Ci/mol) was obtained from Amersham/Searle.

Acrylamide gel electrophoresis. Acrylamide and *N,N'*-methylenebisacrylamide (bisacrylamide) were recrystallized from acetone. *N,N,N',N'*-tetramethylethylenediamine (TEMED) was redistilled. A Tris/glycine buffer system [5], pH 8.1, was employed in both upper and lower reservoirs. In experiments involving various concentrations of small pore gels, the bisacrylamide:acrylamide weight ratio was kept constant at 1 : 30. Electrophoresis and polymerization of the gels were performed at 4° C. Samples were applied to the gels and run at a constant current of 4.5 mA per tube with bromophenol blue as the tracking dye. At the termination of a run, the dye front was marked by inserting a nickel-chromium wire (Chromel, A.H. Thomas Co.).

Enzyme activity was detected in gel columns by immersing the gel in a 2 ml solution containing 2 μmol GTP, 2 mg nitroblue tetrazolium, 0.002 mg phenazine methosulfate and 100 μmol Tris buffer, pH 8.0. The gels were incubated for 30 min at 37° C, rinsed with distilled water and placed in 7% acetic acid to stop the reaction. The purple bands (produced presumably by reduction of nitroblue tetrazolium by D-*erythro*—dihydroneopterin triphosphate) were quantitated with the use of a model 542 Photovolt densitometer equipped with an integrator (model 49). The relative mobilities of the various bands were measured under a dissecting microscope at 5 × magnification.

Sodium dodecylsulfate gel electrophoresis. The methods employed were essentially those outlined by Weber and Osborn [6] with the following modifications: (a) all samples were preincubated for 2 h, at 37° C in the presence of 4 M urea, 1% mercaptoethanol, and 1% sodium dodecylsulfate and (b) 5.4% polyacrylamide gels containing 4 M urea were employed for the electrophoresis.

Preparation of extracts and purification of enzyme. The culture medium and conditions for growth of *L. plantarum* are described in a previous communication [7]. After harvesting, the cells were washed twice in distilled water and lyophilized. 50 g of dried cells were suspended in 250 ml of 0.05 M potassium phosphate buffer, pH 6.8, containing $5 \cdot 10^{-3}$ M EDTA (standard buffer) and 75 g of washed glass beads (0.01 mm Dragen, Werk, George Wild, Bayreuth, Western Germany) were added. The suspension was cooled to 4° C in an ice bath and sonicated (Model W 185, Heat Systems, Ultrasonics, Inc., Plainview, N.Y.) five times at 5-min intervals, allowing enought time between sonications to cool the suspension to 4° C. The sonicate was centrifuged at 27 000 × *g* and the supernatant set aside. The pellet was resuspended in standard buffer

and resonicated. This procedure was repeated twice. The purification procedure, which includes an affinity column step, has been previously reported [8] and results in a purification of approx. 800-fold.

Enzyme assay procedures. The methods employed for the assay of the various cell-free extracts were based on the liberation of radioactive formic acid from [8-^{14}C]GTP and/or increase in absorbance at 330 nm. The formic acid released from [8-^{14}C]GTP was extracted into ethyl acetate as described by Cone and Guroff [9]. This method extracts 40% of the formic acid present in reaction mixtures. Since one-half of this is assayed for radioactivity, the results presented as cpm represent 20% of the total formic acid present in incubation mixtures. The standard reaction mixture contained 0.4 μmol [8-^{14}C]GTP (approx. 275 000 dpm/μmol), 100 μmol of Tris buffer, pH 8.0, or phosphate buffer, pH 6.8, depending upon the enzyme form being studied, and enzyme in a total volume of 400 μl. The reaction mixtures were incubated at 37° C for 15 min. One unit of enzyme is defined as that amount of enzyme capable of liberating 1 nmol of formic acid per 15 min in the standard reaction mixtures.

Sucrose density centrifugation. A Beckman Model L-2-65B ultracentrifuge equipped with an SW-40 swinging bucket rotor was used. Sucrose density gradients, 5—20%, were made in 0.02 M Tris buffer, pH 8.0, or 0.02 M phosphate buffer, pH 8.0, and 2.4 mg of the α form or β form, respectively (Sephadex G-200 purified enzyme fraction) were layered onto the gradients together with 0.25 mg yeast alcohol dehydrogenase as a marker. The centrifugation was performed at 4° C for 16 h at 38 000 rev./min. 9-drop fractions were collected. Alcohol dehydrogenase was assayed in a total volume of 750 μl. 25 μl of the fraction were added to a reaction mixture containing 250 μl of 0.03 M sodium pyrophosphate buffer, pH 8.8, 50 μl 95% ethanol and 50 μl of 0.012 M NAD. The reaction was followed for 2 min at 340 nm. Dihydroneopterin triphosphate synthetase was assayed in a 200-μl reaction mixture containing 0.4 μmol [8-^{14}C]GTP (278 775 dpm/μmol) and 100 μl of the sucrose fraction. Incubation was at 37° C for 3 h. The molecular weights and sedimentation constants were calculated according to the method of Martin and Ames [10].

Miscellaneous materials and methods. Protein was determined by the method of Lowry et al. [11] or by the method of Warburg and Christian [12]. Radioactivity was determined with a Packard model 3375 scintillation spectrometer using 10 ml of a modified Bray's [13] scintillation fluid.

Results

Separation of multiple forms by polyacrylamide gel electrophoresis and DEAE-cellulose chromatography

Purification of the dialyzed affinity column-treated enzyme fraction [8] by DEAE-cellulose chromatography resulted in an elution pattern as depicted in Fig. 1. Enzyme was applied to the DEAE-cellulose column in order to effect a profile as reported previously [1]. The elution pattern gave two peaks of enzyme activity. Polyacrylamide gel electrophoresis of peak II gave a single protein band when stained with Coomassie blue (Fig. 2). This electrophoretic form has been designated the β form and is active when gels are incubated in the enzyme-staining solution described in Materials and Methods. Peak I gave

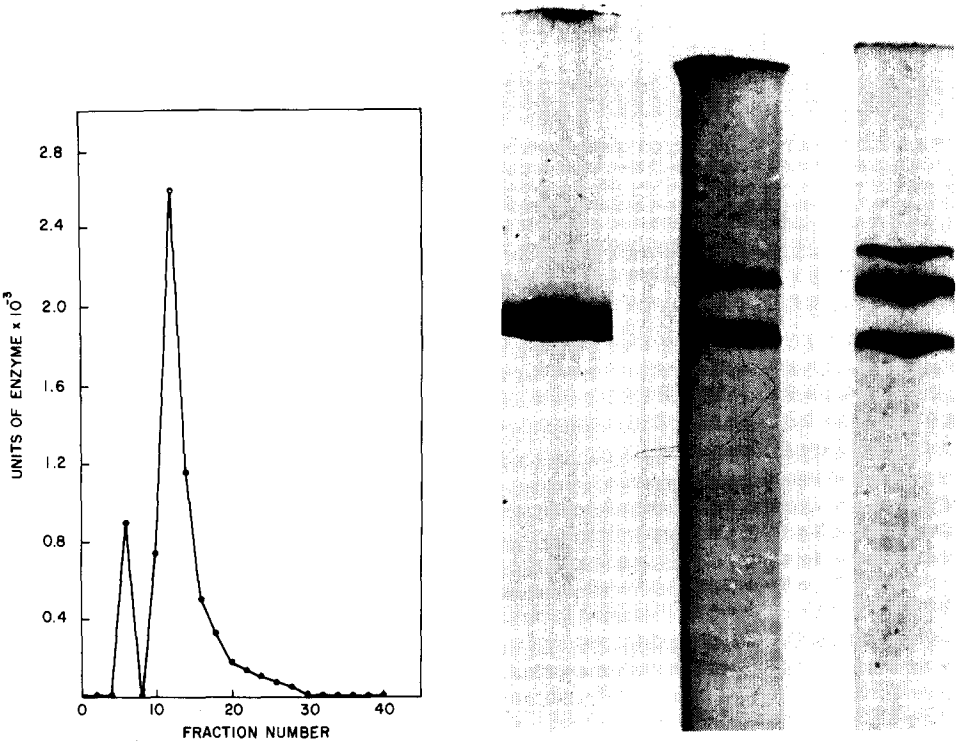


Fig. 1. DEAE-cellulose chromatography of affinity column-treated enzyme. Approx. 8.5 ml (28.2 mg protein) of a dialyzed affinity column-treated enzyme fraction [8] was applied to a DEAE-cellulose column (1 × 10 cm) and eluted with a linear gradient composed of 100 ml of 0.02 M phosphate buffer, pH 6.0, containing $5 \cdot 10^{-3}$ M EDTA in the mixing flask and 100 ml of the same buffer plus 0.35 M KCl in the reservoir. 2 ml fractions were collected and monitored for enzyme activity.

Fig. 2. Demonstration of multiple enzyme forms of D-*erythro*-dihydroneopterin triphosphate synthetase by gel electrophoresis. The gel on the left depicts the electrophoretic pattern obtained when approx. 40 μg of protein from the pooled peak II fraction from the DEAE-cellulose chromatography (Fig. 1) was subjected to electrophoresis in 7% polyacrylamide. This is the most electronegative of the various enzyme forms and has been designated the β enzyme form. The gel in the middle of the figure is a mixture of the α and β enzyme species, while the gel on the right depicts the α′, α and β enzyme forms respectively. Electrophoresis was performed from top to bottom. Following electrophoresis, the gels were stained overnight in 0.05% Coomassie blue in 12.5% trichloroacetic acid followed by destaining in 10% trichloroacetic acid.

two protein bands upon electrophoresis in 7% polyacrylamide, both of which exhibited enzymatic activity when incubated in the enzyme-staining solution. The two bands (Fig. 2, middle gel) have been designated the α and β forms of the enzyme based on their relative electronegative properties. The more electronegative species is the β form. If electrophoresis was performed immediately following chromatography only the α band was detected, whereas if the mixture was allowed to stand overnight in 0.05 M phosphate buffer, pH 6.0, containing $1 \cdot 10^{-3}$ M EDTA, the α form disappeared and there was a corresponding increase in the amount of the β band detected. This suggested that the α form was converted to the β form upon standing in phosphate buffer. This is further supported by evidence given in later portions of the text. Fig. 2 (right

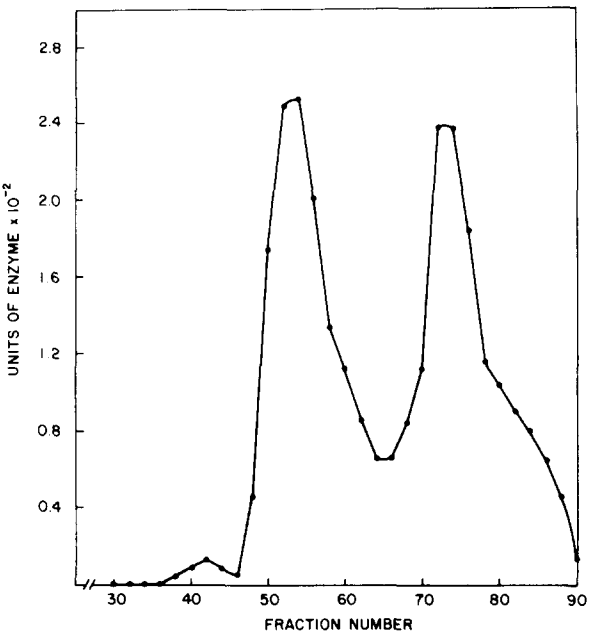


Fig. 3. DEAE-cellulose chromatography of the purified enzyme. A purified enzyme preparation (approx. 1.5 mg protein) was applied to a 2.5 × 30 cm Sephadex G-25 column equilibrated with 0.01 M Tris, pH 8.0, in order to convert the β enzyme form to the α form. The mixture was subsequently applied to a 1 × 18 cm DEAE-cellulose column equilibrated with 0.01 M Tris buffer, pH 8.0. Linear gradient elution was performed with 100 ml 0.01 M Tris, pH 8.0, in the mixing flask and 100 ml of the same buffer plus 0.35 M KCl in the reservoir. 2-ml fractions were collected and assayed for enzyme activity. The initial peak contained only the purified α enzyme form while the second peak contained a mixture of the α and β enzyme forms.

gel) depicts a third enzyme species designated the α form, which is found mixed with the α form in partially or highly purified enzyme preparations which have been passed through a 2.5 × 30 cm Sephadex G-25 column equilibrated with 0.01 M Tris buffer, pH 8.0, followed by dialysis for 16 h against the same buffer. The gel was made by mixing a preparation of pure β form with a mixture of α and α' forms in order to demonstrate the relative mobilities of each of the enzyme species in 7% polyacrylamide. Although these gels were stained only for protein all three forms may also be detected by the enzyme-staining procedure. Since we have been routinely unable to separate the α' form from the α form by DEAE chromatography we have worked primarily with the α or β species of enzyme. Sephadex G-25 chromatography of purified enzyme preparations in 0.01 M Tris buffer, pH 8.0, results in a mixture of these enzyme forms. The α species may be separated from the β enzyme species by DEAE-cellulose chromatography employing a salt gradient in 0.01 M Tris buffer, pH 8.0. The elution pattern under these conditions is shown in Fig. 3. The initial peak contained the α enzyme form whereas the second peak contained a mixture of the α and β species. Since electrophoresis was performed some hours following chromatography, it is likely that conversion of β to α form occurred in the eluted fractions following chromatography.

Heat inactivation of the α and β enzyme forms

Heat inactivation studies of the α and β enzyme forms revealed that the β species is the more stable of the two enzyme forms. Exposure of the α form to a temperature of 65°C for 15 min led to a 54% loss in enzymic activity when compared to unheated enzyme preparations whereas the β species retained 85% of its original activity under identical conditions.

Molecular weight estimations of the enzymic species

The molecular weight of the α and β forms was estimated by sucrose density centrifugation with alcohol dehydrogenase as a marker. The molecular weight of the β species was estimated as 207 300 (Fig. 4B). The integrity of the enzyme form was confirmed following centrifugation by polyacrylamide gel electrophoresis. In the case of the purified α form, however, approx. 50% of the enzyme was converted to the α′ species during the 16-h centrifugation period. However only a single peak of enzyme activity was detected (Fig. 4A) and a molecular weight of 198 500 was determined for the enzyme species. A mixture of the α and β forms centrifuged in a 5—20% sucrose density gradient (0.02 M Tris buffer, pH 8.0) gave a single peak of activity and a molecular weight of 202 350 (data not shown). Gel electrophoresis demonstrated only the α and α′ enzyme species indicating that the β form was converted to the α form and a portion of the α form converted to the α′ form during centrifugation. That the molecular weights of each of the enzyme species are similar is

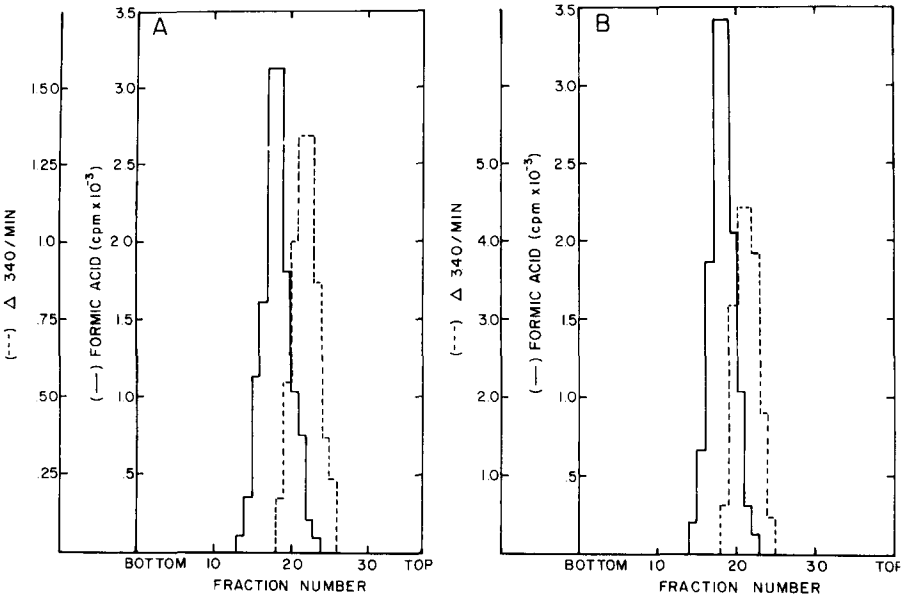
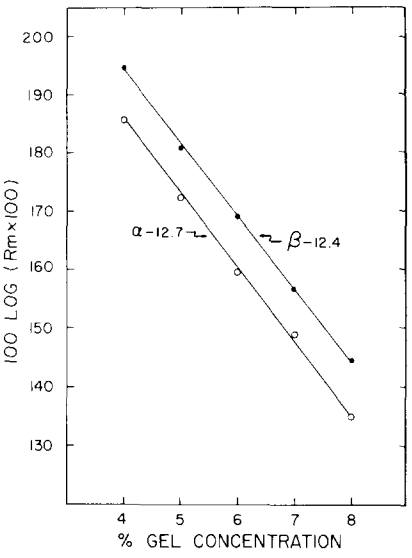


Fig. 4. Sucrose density centrifugation of the α and β enzyme forms. (A) Depicts the sucrose density profile obtained when the α enzyme form was centrifuged in a sucrose density gradient made in 0.02 M Tris buffer, pH 8.0. Yeast alcohol dehydrogenase was employed as a marker. A single peak of enzyme activity was resolved upon assay of the gradient fractions corresponding to a molecular weight of 198 350. (B) Demonstrates the profile obtained when the β enzyme form was centrifuged in a similar gradient made in 0.02 M phosphate buffer, pH 8.0. The molecular weight of this species was estimated as 207 300.



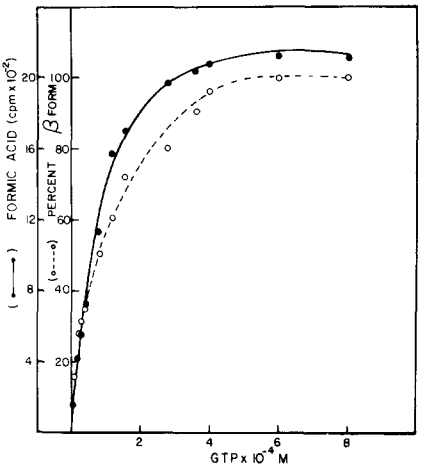


Fig. 5. Ferguson plot of a mixture of α and β enzyme forms. A mixture of the α and β enzyme species was subjected to electrophoresis in various concentrations of polyacrylamide. The slopes of the regression lines indicate that the two lines are parallel.

Fig. 6. Rate of the enzymatic reaction as a function of GTP concentration and the amount of β form present in the reaction mixtures. Reaction mixtures containing approx. 10 units of partially purified α form were prepared in 0.1 M Tris buffer, pH 8.0, and the various concentrations of GTP shown in the graph. Incubations were at 37°C for 15 min. Following the incubation period, an aliquot of each of the reaction mixtures was immediately subjected to electrophoresis in 7% polyacrylamide, the gel stained for enzyme activity and the relative amount of each enzyme form estimated by densitometry.

further supported by data obtained by plotting the logarithm of the relative mobility versus the percent acrylamide (i.e. Ferguson plots [14,15]). As shown in Fig. 5, parallel lines were obtained for the α and β forms, indicating that the species differ in charge or conformation rather than molecular weight.

The above data are consistent with the hypothesis that three conformational states of the enzyme exist. The β conformation may be converted to the α form by removal of phosphate and this form may subsequently be converted to the α′ form. The conversions are readily reversible (i.e. $\alpha' \rightarrow \alpha \rightarrow \beta$) by the addition of phosphate or substrate (see below).

Dependence of enzymatic activity on enzyme conformation

Fig. 6 presents data which were obtained with partially purified extracts containing the α form of the enzyme. Reaction mixtures consisted of 10 units of a partially purified preparation of the α enzyme form prepared in 0.1 M Tris · HCl buffer, pH 8.0. Substrate was added at various concentrations and the amount of formic acid liberated after a 15-min incubation at 37°C plotted against GTP concentration. Also plotted in Fig. 6 is the percentage of β form produced following incubation of the α form with the various concentrations of substrate. The relative amount of each of the two forms was estimated after polyacrylamide gel electrophoresis of each of the reaction mixtures. Quantitation involved densitometry of the gels following incubation in the enzyme-

staining solution. Under the conditions employed the amount of formazan produced was linear with the amount of enzyme protein present. Since the maximum velocity of reaction mixtures originally containing the α enzyme form exhibited approx. 60% of the maximum velocity of reaction mixtures containing an equal quantity of the β enzyme form (see below) and since the amount of α form was quantitated on the basis of enzyme activity (i.e. formazan production), a correction factor was applied in order to determine the amount of α form in relation to the quantity of β form present in the gels. As shown in the figure, there appears to be a correlation between the amount of formic acid liberated and the amount of β form present in reaction mixtures originally containing only the α form of the enzyme.

Lineweaver-Burk plots of the kinetic data obtained with the α or β enzyme species indicated that both forms had identical apparent K_m values ($1 \cdot 10^{-4}$ M) in 0.1 M Tris buffer, pH 8. However, the V value of the reaction mixtures initially containing the α enzyme species was consistently 60—64% of that of the β conformer. Thus, the kinetics are similar to those of non-competitive inhibition and are consistent with the hypothesis that the α conformer is a catalytically inactive molecule. Addition of substrate to reaction mixtures containing the α species apparently induces a concentration and time-dependent conversion of the inactive molecule to the functional, enzymatically active β conformation. The apparent activity of the α and α' enzyme forms in polyacrylamide gels after exposure to the enzyme-staining solution is due to the conversion of these forms to the enzymatically active β conformer after exposure of the gel to the substrate, GTP.

Under optimal assay conditions the apparent K_m of the purified β conformer in 0.1 M phosphate buffer, pH 6.8, was $2.48 \cdot 10^{-5}$ M. In addition to substrate effecting the conformational transition of the α to β enzyme form, gel electrophoresis demonstrated that the addition of phosphate buffer to reaction mixtures containing the α conformer also led to the formation of the enzymatically active molecule. Moreover, incubation of the α form in 0.05 M phosphate buffer for 1 min prior to the addition of substrate led to V and K_m values similar to reaction mixtures originally containing the β conformer. Nucleotides other that the substrate including GDP, ATP, and ADP have also been demonstrated to induce the transition of α to β configuration at concentrations of $1 \cdot 10^{-3}$ M. However, GMP, AMP or guanosine at concentrations up to $2.5 \cdot 10^{-3}$ M had no detectable effect on the α enzyme form. The nucleotides effecting the transition from the inactive to active enzyme form neither serve as substrates nor as inhibitors of the enzymic reaction.

Additional evidence for the contention that the β configuration of the enzyme is necessary for catalytic activity is presented in Fig. 7. In order to investigate the properties of the α and β enzyme forms in the presence of $6 \cdot 10^{-4}$ M GTP, reaction mixtures containing either the α or β form were incubated in an ice bath for periods of up to 1 h. As shown in the figure, reaction mixtures containing the β form exhibit a straight line when the amount of formic acid liberated is plotted against time. However, in the case of reaction mixtures initially containing only the α configuration, a pronounced lag period of up to 20 min was observed before formic acid liberation could be detected. At the end of the 60-min incubation period, reaction mixtures initial-

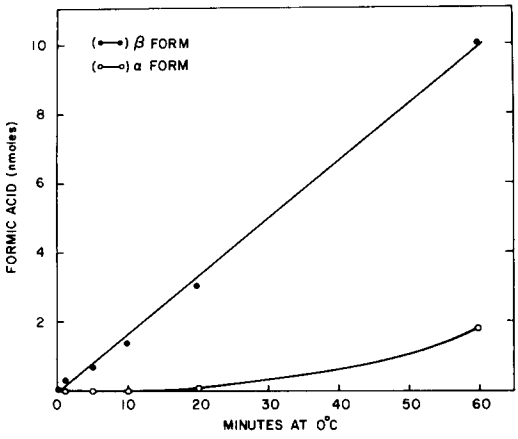


Fig. 7. Reaction rates of the α and β conformers at 0° C. Partially purified α or β enzyme species (50 units) was added to 1.6 μmol [8-^{14}C]GTP, containing 0.1 M Tris, pH 6.5, in a total volume of 2.4 ml. Incubation of the reaction mixtures was carried out at 0° C. At the intervals depicted in the figure, 0.4 ml of each reaction mixture was removed and extracted with ethyl acetate to determine the amount of formic acid liberated.

ly containing the α form exhibited only 18% of the activity of similar reaction mixtures containing the β conformer. At low temperature, the rate of the substrate-dependent conversion from the inactive to catalytically active conformer is extremely slow and thus results in a dramatic lag in [^{14}C]formic acid release. Since the conformational transition is not required in reaction mixtures containing the β conformer, formic acid release is linear with time.

Effect of ions on enzyme activity

In order to determine whether various anions or cations might influence formic acid release from GTP, the α and β forms of the enzyme were tested in reaction mixtures containing various anions or cations at a final concentration of $2.5 \cdot 10^{-3}$ M. Particularly interesting was the differential inhibition of reactions mixtures containing the α enzyme species and $MgSO_4$, $MgCl_2$ or $CoSO_4$. The activity of reaction mixtures containing these ions compared to controls without ions indicated that $MgSO_4$ inhibited the activity of reaction mixtures containing the α enzyme conformer 49.5%, $MgCl_2$ by 47% and $CoSO_4$ by 40%. In contrast to these results, the β conformer was inhibited less than 9% by the same concentration of ions.

To further investigate this phenomenon, the α form of the enzyme was incubated with $2.5 \cdot 10^{-3}$ M $MgSO_4$, $6 \cdot 10^{-4}$ M GTP, in 0.1 M Tris buffer, pH 8.0. Incubation was at 37° C for 15 min. After incubation, the reaction mixtures were subjected to electrophoresis in 7% polyacrylamide gels. The controls consisted of an identical reaction mixture in which $MgSO_4$ was omitted. Following electrophoresis, the amount of α or β form was estimated by incubation of the gels in the standard enzyme-staining solution and were subsequently scanned with the densitometer to estimate the amount of each of the enzyme species. 94% of the enzyme was detected as the β form in reaction mixtures without added $MgSO_4$. In reaction mixtures containing $MgSO_4$, however, only

52.7% of the enzyme was converted to the β species. These results are in agreement with the magnesium inhibition data based on [^{14}C]formic acid release and further support the contention that the α enzyme form is an enzymatically inactive conformer of the β enzyme species. The results indicate that Mg^{2+} or the Mg^{2+}-GTP complex interferes with the conversion of the α conformer to the β conformer. Assuming a dissociation constant of $1.8 \cdot 10^{-4}$ M for Mg^{2+}-GTP [16], the amount of free GTP may be calculated as $5 \cdot 10^{-5}$ M. This concentration of free GTP would under the previously described conditions (Fig. 6), be sufficient to convert approx. 40—50% of the α conformer to the β enzyme species. The addition of $2.5 \cdot 10^{-3}$ M Mg^{2+} to reaction mixtures containing GDP, ATP or ADP also inhibited the α to β transition, but the inhibition was less effective with the nucleotide diphosphate which may reflect the lower binding affinity of magnesium for these compounds [16]. Magnesium did not affect the rate of conversion in the presence of 0.05 M phosphate buffer.

The subunit structure of the purified β conformer

The subunit structure of the purified enzyme was investigated by procedures previously outlined by Weber and Osborn [6] employing sodium dodecylsulfate and polyacrylamide gel electrophoresis. A single protein band with an estimated molecular weight of 20 000 was obtained following sodium dodecylsulfate treatment (Fig. 8). If one assumes a molecular weight of approx.

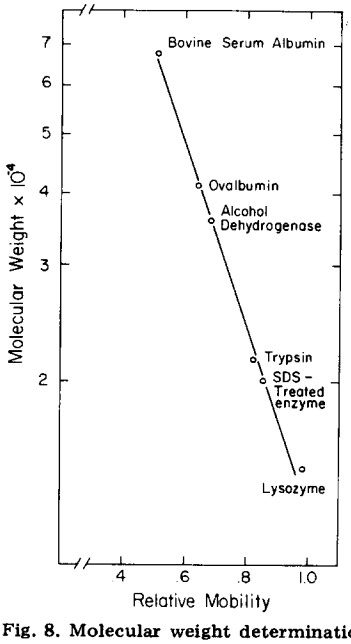


Fig. 8. Molecular weight determination of dissociated β conformer by sodium dodecylsulfate gel electrophoresis. The protein standards and purified enzyme were incubated in 4 M urea, 1% sodium dodecylsulfate and 1% mercaptoethanol in 0.01 M sodium phosphate buffer, pH 7.0, for 2 h at 37° C. Following incubation 28 μg of each of the protein standards and 22 μg of the purified enzyme were applied to 5.4% polyacrylamide gels containing 4 M urea. Electrophoresis was performed at 8 mA per tube. Gels were stained with Coomassie blue.

200 000 for the enzyme, the enzyme would seem to be comprised of 10 polypeptide chains of similar molecular weights. It has not yet been determined whether these polypeptides are identical in amino acid composition.

Investigation of the regulatory properties of the α and β conformers

A variety of compounds were tested as possible feedback inhibitors of dihydroneopterin triphosphate synthetase. Both the α and β forms of the enzyme were tested for inhibition of formic acid release by preincubation of each of the enzyme forms for 10 min in the presence of $4.4 \cdot 10^{-4}$ or $1 \cdot 10^{-3}$ M of the test compound. Compounds tested for inhibitory activity include folate, dihydrofolate, dihydropteroyltriglutamate, N^5-formyltetrahydrofolate, riboflavin, riboflavin 5′-phosphate, methionine, deoxy GTP, dihydropteroate and D-*erythro*-dihydroneopterin. None of the compounds tested proved to be inhibitory to enzymatic formic acid release.

In order to determine if formic acid and/or riboflavin control enzyme synthesis via repressive effects, cells were cultured in a defined medium [7] in the presence or absence of riboflavin and/or folic acid. No significant changes in the specific activity of the enzyme was observed in crude extracts of cells cultured under any of the test conditions.

Discussion

Multiple enzyme forms may be grouped into two distinct classes based upon whether the multiple forms are genetic or non-genetic variants of a specific enzyme molecule [17]. Multiple enzyme forms which differ due to genetically determined differences in primary structure are termed isoenzymes [18] of which lactate dehydrogenase [19], tyrosinase [20], and adenosine deaminase [21] are but a few examples of the number of enzymes found in this category.

Non-genetic multiple enzyme forms are proteins which possess identical primary amino acid sequences but may differ in the amount of ligand bound to the enzyme [22] or in aggregation states of identical subunits [23]. The studies of Teipel and Koshland [24,25] involving reassociation of various denatured enzymes have shown that environmental influences such as ionic strength, the presence of substrate, cofactors or salts play an important role in the refolding of polypeptide chains and thus influence the proportion of active and inactive conformational states of otherwise identical protein molecules.

The data obtained with D-*erythro*-dihydroneopterin triphosphate synthetase are consistent with the idea that the conformational structure of the enzymatically active β enzyme species is stabilized by non-covalently bound phosphate ions or certain nucleotides. Removal of these compounds by dialysis or Sephadex G-25 chromatography induces a conformational shift to the metastable α and α prime forms which may be distinguished on the basis of their relative mobilities in polyacrylamide gels. Phosphate ions are known to stabilize other enzymes and in the case of rabbit muscle lactate dehydrogenase [26] not only stabilize the tetrameric structure of the enzyme but also seem to activate coenzyme binding sites on the molecule.

Our data suggest that the three enzyme forms are interconvertible, are of

similar molecular weights and are composed of approx. 10 subunits each of which has a molecular weight of 20 000. While amino acid analyses have not been performed, it seems reasonable to suggest that the enzyme forms are non-genetic variants and are conformers of a single protein molecule.

Kinetic as well as electrophoretic studies with the α and β enzyme forms support the idea that the α (and probably the α prime) conformer is an enzymatically inactive species which must be converted to the β conformer before enzymatic activity can be detected. Unlike true substrate activation, however, the conformational change of the α form to β form is not limited to the substrate GTP. Other molecules including GDP, ATP, ADP and phosphate effect this transition.

The failure to demonstrate feedback inhibition or repression of the synthetase does not exclude the possiblity of strict regulation of enzyme activity. The observation that magnesium-nucleotide complexes inhibit the conversion of the inactive to enzymatically active conformer suggests the possibility of control being exercised via changes in the intracellular concentration of free and complexed nucleotides. A similar mechanism has been invoked by Penner and Cohen [27] for controlling the rate of fumarate biosynthesis. In addition, structural transitions induced by ligand binding are known to affect the rate of enzyme inactivation by proteolytic enzymes [28] as well as possible intracellular binding sites [29] and thus may influence enzymatic activity in vivo.

Studies similar to those performed with the α enzyme form have been hampered in the case of the α' conformer by our inability to separate the two forms by various column chromatographic methods. It seems likely, however, that the α' conformer is also enzymatically inactive since it is derived from the α form and undergoes conformational changes to the β conformer upon the addition of substrate, nucleotides or phosphate.

The precise molecular interactions of phosphate and nucleotides with D-*erythro*-dihydroneopterin triphosphate synthetase and the location of these sites must await detailed analysis of the primary, secondary and tertiary structure of the protein.

Multiple forms of GTP cyclohydrolase have been reported in two other microorganisms. Cone et al. [30] demonstrated by sucrose density centrifugation that the enzyme from a *Comamonas* sp. may exist in two molecular weight forms depending upon the concentration of the suspending buffer. In 5 mM Tris buffer the molecular weight of the enzyme was estimated by gel filtration to be approx. 650 000. The molecular weight of the smaller enzyme species in 50 mM Tris buffer was not determined. Elstner and Suhadolnik [31] reported that a GTP cyclohydrolase preparation from *Streptomyces rimosus* could be separated into two protein fractions following dialysis against 50 mM Tris buffer and DEAE-cellulose chromatography. However, the enzyme fractions were not further characterized and thus it is not clear whether these fractions represent enzymatically active subunits or whether other factors are responsible for the observed polymorphism.

Acknowledgement

This work was supported by National Science Foundation grant GB-35538.

References

1 Jackson, R.J. and Shiota, T. (1971) J. Biol. Chem. 246, 7454—7459
2 Burg, A.W. and Brown, G.M. (1968) J. Biol. Chem. 243, 2349—2358
3 Plowman, J., Cone, J.C. and Guroff, G. (1974) J. Biol. Chem. 249, 5559—5564
4 Shiota, T., Disraely, M.N. and McCann, M.P. (1964) J. Biol. Chem. 239, 2259—2266
5 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
6 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
7 Shiota, T. (1959) Arch. Biochem. Biophys. 80, 155—161
8 Jackson, R.J., Wolcott, R.M. and Shiota, T. (1973) Biochem. Biophys. Res. Commun. 51, 428—435
9 Cone, J. and Guroff, G. (1971) J. Biol. Chem. 246, 979—985
10 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—421
13 Bray, G.A. (1960) Anal. Biochem. 1, 279—285
14 Ferguson, K.A. (1964) Metabolism 13, 985—1002
15 Hedrick, J.L. and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155—164
16 O'Sullivan, W.J. and Perrin, D.D. (1961) Biochim. Biophys. Acta 52, 612—614
17 Kaplan, N.O. (1968) Ann. N.Y. Acad. Sci. 151, 382—399
18 IUPAC-IUB Commission on Biochemical Nomenclature (1971) J. Biol. Chem. 246, 6127—6128
19 Markert, C.L. and Massaro, E.J. (1968) Science 162, 695—697
20 Jolley, Jr, R.L., Nelson, R.M. and Robb, D.A. (1969) J. Biol. Chem. 244, 3251—3257
21 Spencer, N., Hopkinson, D.A. and Harris, H. (1968) Ann. Hum. Genet. 32, 9—14
22 Marangos, P.J. and Constantinides, S.M. (1974) J. Biol. Chem. 249, 951—954
23 Frieden, C. (1963) Biochem. Biophys. Res. Commun. 10, 410—415
24 Teipel, J.W. and Koshland, D.E. (1971) Biochemistry 10, 792—798
25 Teipel, J.W. and Koshland, D.E. (1971) Biochemistry 10, 798—805
26 Lovell, S.J. and Winzor, D.J. (1974) Biochemistry 13, 3527—3531
27 Penner, P.E. and Cohen, L.H. (1969) J. Biol. Chem. 244, 1070—1075
28 Yang, S.T. and Deal, Jr, W.C. (1969) Biochemistry 8, 2814—2820
29 Masters, C.J., Sheedy, R.J., Winzor, D.J. and Nichol, L.W. (1969) Biochem. J. 112, 806—808
30 Cone, J.E., Plowman, J. and Guroff, G. (1974) J. Biol. Chem, 249, 5551—5558
31 Elstner, E.F. and Suhadolnik, R.J. (1971) J. Biol. Chem. 246, 6973—6981