Biochimica et Biophysica Acta, 334 (1974) 448–458

BBA 67149

EFFECTS OF TEMPERATURE AND AMMONIUM IONS ON FORMYLTETRAHYDROFOLATE SYNTHETASE FROM *CLOSTRIDIUM THERMOACETICUM**

W. THOMAS SHOAF, SUE H. NEECE and LARS G. LJUNGDAHL
Department of Biochemistry, University of Georgia, Athens, 30602 (U.S.A.)
(Received October 1st, 1973)

SUMMARY

NH_4^+ and K^+ but not Na^+ increase the thermostability of formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3) from the thermophile *Clostridium thermoaceticum*. When the reaction is measured in the direction of synthesis of 10-formyltetrahydrofolate (forward direction), NH_4^+ and K^+ activate the enzyme at low but not at high concentrations of formate. The apparent K_m of formate is decreased 10-fold by the presence of 2 mM NH_4Cl. A small decrease in the apparent K_m of ATP and tetrahydrofolate also occurs on addition of NH_4Cl. The V of the reaction is not changed. The reverse reaction is stimulated 4-fold by the addition of 20 mM NH_4Cl. Arrhenius plots show that the activation energy for the synthesis of 10-formyltetrahydrofolate is higher below 40–43 °C than above. Plots of apparent K_m values for each of the substrates (for the forward reaction) versus temperature, have breaks at 40–43 °C in the presence or absence of NH_4Cl. The fluorescence (at 325 nm) of the enzyme in the absence of NH_4Cl decreases linearly with increasing temperature; however, in the presence of NH_4Cl, the linearly decreasing fluorescence has a slope change at 40 °C. Absorbance of the enzyme at 295 nm increases linearly with increasing temperature with a slope change at 40 °C both in the presence and absence of NH_4Cl. The kinetic and physical data indicate that the enzyme undergoes a temperature-dependent conformational change at 40–43 °C.

INTRODUCTION

Formyltetrahydrofolate synthetase (EC 6.3.4.3) catalyzes the following reaction:

tetrahydrofolate + formate + ATP $\rightleftarrows$ 10-formyltetrahydrofolate + ADP + P_i (1)

The enzyme has been isolated from a large number of micro-organisms, plants and animals [2]. The enzyme in *Clostridium cylindrosporum* and *Clostridium acidiurici* has been crystallized and extensively studied [3, 4]. These bacteria ferment purines

* A preliminary report of parts of this investigation has been published [1].

and 10-formyltetrahydrofolate is an intermediate in the fermentation. It is likely that the reverse of Reaction 1, is one of the principal ways of forming ATP in the purine-fermenting bacteria [4].

Formyltetrahydrofolate synthetase has also been isolated from *Clostridium thermoaceticum* [5] and demonstrated in *Clostridium formicoaceticum* [6]. Both bacteria ferment hexoses to acetate. During the fermentation CO_2 is reduced to acetate in a pathway with formate and one-carbon derivatives of tetrahydrofolate as intermediates [7]. The physiological role of the enzyme is to catalyze the formation of 10-formyltetrahydrofolate, which then is reduced to 5-methyltetrahydrofolate and is converted to acetate [8]. The enzyme from *C. thermoaceticum* has been crystallized, but in contrast with the enzymes from *C. cylindrosporum* and *C. acidiurici* the crystals of *C. thermoaceticum* enzyme are enzymatically inactive (Neece, S. H. and Ljungdahl, L. G., unpublished).

The *C. cylindrosporum* and *C. thermoaceticum* enzymes are remarkably similar. Both have a molecular weight of 240 000 [9, 10] and consist of four subunits, which apparently are identical in respective enzymes [4, 5]. They have in common also several other properties. However, the *C. thermoaceticum* enzyme is more heat stable than the enzyme from *C. cylindrosporum*. Furthermore, the *C. thermoaceticum* enzyme does not require NH_4^+ or K^+ for activity at any temperature. The enzyme of *C. cylindrosporum* requires NH_4^+ or K^+ for activity at 37 °C, but not at 20 °C [11] and dissociates to monomers on removal of the monovalent cations [12]. Such a dissociation has not been observed with the *C. thermoaceticum* enzyme. However, NH_4^+ or K^+ affect the *C. thermoaceticum* enzyme, and in this communication, we report that these ions increase the thermostability and alter some of the kinetic and physical properties of the enzyme.

MATERIALS AND METHODS

Assay of formyltetrahydrofolate synthetase: forward reaction

The enzyme was assayed essentially as described by Rabinowitz and Pricer [3]. Routine assays contained 100 mM maleate (Tris), pH 8, 10 mM $MgCl_2$, 5 mM ATP (Tris), 2 mM (−)-tetrahydrofolate, 200 mM mercaptoethanol, 2 mM NH_4Cl, and 40 mM formate (Tris), pH 8, in a total volume of 0.5 ml. Unless otherwise indicated the incubation time was 10 min at 50 °C. 1 ml of 0.36 M HCl was added to stop the reaction, and convert the product, 10-formyltetrahydrofolate to 5,10-methenyltetrahydrofolate. This compound was determined spectrophotometrically by absorbance at 350 nm ($\varepsilon = 24\,900\ M^{-1} \cdot cm^{-1}$). 1 unit of enzyme is that amount which catalyzes the formation of 1 μmole of product per min under the above conditions.

Assay of formyltetrahydrofolate synthetase: reverse reaction

The reverse reaction was followed by measuring the disappearance of 10-formyltetrahydrofolate. Routine assays contained 200 mM Tris (Cl), pH 8, 2 mM $MgCl_2$, 1 mM ADP (Tris), 200 mM mercaptoethanol, 40 mM phosphate (Tris), pH 8, 20 mM NH_4Cl and 0.35 mM (±)-10-formyltetrahydrofolate in a total volume of 0.25 ml. The incubation time was 10 min at 50 °C. The reaction was terminated by the addition of 1 ml 0.36 M HCl, and 10-formyltetrahydrofolate was determined spectrophotometrically as described for the forward reaction.

Purification of formyltetrahydrofolate synthetase from C. thermoaceticum

The purification procedure was essentially that reported by Ljungdahl et al. [5] followed by reversed flow chromatography on a Sephadex G-200 column (2.5 cm × 100 cm). The column was equilibrated and eluted with 0.1 M maleate (K^+), pH 7. The activity of the enzyme was 250 units/mg of protein.

Protein assay

Protein concentrations were determined by trichloroacetic acid turbidity assays using bovine serum albumin as a standard.

Chemicals

Tetrahydrofolate was prepared as previously described [6]. 10-Formyltetrahydrofolate was prepared from tetrahydrofolate as described by Rowe [13]. Mercaptoethanol, bovine serum albumin, Tris, and the Tris salt of ATP and ADP were obtained from Sigma Chemical Co.

RESULTS

Stability of tetrahydrofolate at high temperatures

Tetrahydrofolate is not stable in the absence of reducing conditions, especially at high temperatures. However, it was established that a solution of tetrahydrofolate (5 mM) is completely stable in 0.21 M mercaptoethanol for eight minutes at 60 °C and pH 7.6 and that only a slight loss occurs in 0.11 M mercaptoethanol. At 50 °C and in 0.16 M mercaptoethanol, the loss of tetrahydrofolate (0.16 mM) is insignificant (pH 8.0) during a 12-min period.

Protection of synthetase by NH_4Cl from heat inactivation and inactivation by p-hydroxymercuribenzoate

The effect of NH_4Cl on the heat inactivation of the enzyme is shown in Fig. 1. In the absence of NH_4Cl, the enzyme is rapidly inactivated at 70 °C. In the presence of 50 mM NH_4Cl, the enzyme loses activity only slowly. After approximately 15 min, half of the initial activity remains and there is no more loss of activity as if the enzyme had undergone a change to a more stable form. The original activity could not be

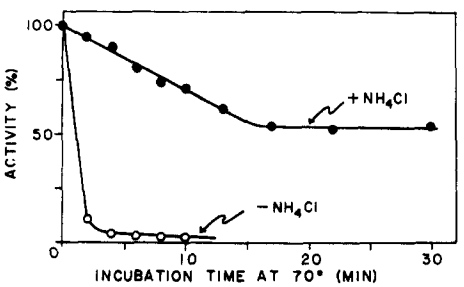

Fig. 1. Protection of formyltetrahydrofolate synthetase from heat inactivation by NH_4Cl. Formyltetrahydrofolate synthetase (2.6 μg/ml) was incubated at 70 °C in 0.1 M maleate (Tris), pH 8, containing 0.05 M mercaptoethanol. Incubations were with and without 0.05 M NH_4Cl. Aliquots were taken at the indicated times and assayed at 50 °C in the forward direction as described in Materials and Methods.

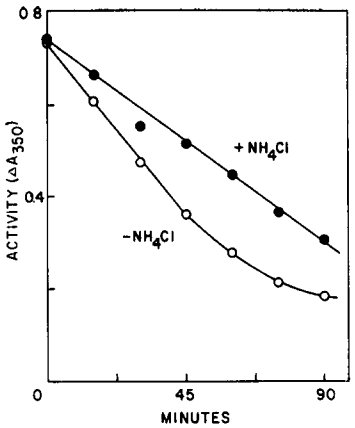

Fig. 2. Inactivation of formyltetrahydrofolate synthetase by *p*-hydroxymercuribenzoate in the presence and absence of NH_4Cl. The enzyme was diluted to approximately 0 8 μg/ml in 0.1 M Tris–HCl at pH 8 plus or minus 2 mM NH_4Cl and containing 2 mM *p*-hydroxymercuribenzoate at 50 °C.

recovered by reducing the temperature. The heat treated enzyme has the same apparent K_m values for all the substrates at 50 °C as the non-heated form in the presence of NH_4Cl.

The enzyme is inactivated by *p*-hydroxymercuribenzoate. This inactivation is less rapid in the presence of NH_4^+ than in their absence as is seen in Fig. 2.

Activation of the forward reaction by NH_4^+

The enzyme activity is stimulated by NH_4Cl or KCl and to a lesser degree, NaCl (Fig. 3)*. The magnitude of activation by NH_4Cl is markedly dependent on the concentration of the substrate formate (Fig. 4A). At high formate concentrations, the addition of NH_4Cl has almost no affect on enzyme activity. Thus the slope of the

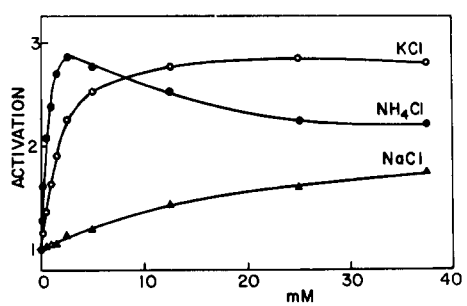

Fig. 3. Activation of formyltetrahydrofolate synthetase by monovalent cations. The enzyme ($6 \cdot 10^{-3}$ unit) was assayed in the presence of varying concentrations of monovalent cations as indicated using an assay mixture containing 100 mM maleate (Tris), pH 8, 10 mM $MgCl_2$, 5 mM ATP (Tris), 40 mM formate (Tris), 1.6 mM tetrahydrofolate, and 160 mM mercaptoethanol.

* We initially reported that the synthetase from *C. thermoaceticum* was not activated by NH_4^+ [5]. These experiments show that this is correct at high formate concentrations, but enzyme activity is increased by NH_4^+ at lower concentrations of formate.

line in Fig. 4A at high formate concentrations becomes zero. Thus the specific activity cannot be increased by addition of NH_4Cl. However at low concentrations of formate (1.2 mM) the enzyme activity can be increased 23-fold by addition of NH_4Cl to a concentration of 5 mM. KCl activates in a similar manner.

In the absence of NH_4^+, the apparent K_m of formate is about 50 mM (Fig. 4B). The addition of 2 mM NH_4Cl results in a 10-fold decrease in the apparent K_m for formate.

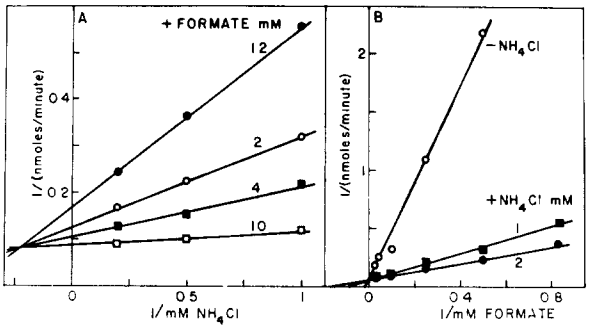

Fig. 4. Lineweaver–Burke plots for formate and NH_4Cl. The enzyme ($6 \cdot 10^{-3}$ unit) was assayed at 50 °C as described in Fig 3, but with the concentrations of formate and NH_4Cl as indicated. A is a double reciprocal plot with NH_4Cl as variable, and B, with formate as the variable.

The apparent K_m values for tetrahydrofolate and ATP were determined at 50 °C in the presence of saturating concentrations of formate and the other non-variable substrate in the presence and in the absence of NH_4^+. As with formate as the variable substrate, no change in V was observed with either tetrahydrofolate or ATP. However, a small decrease of the apparent K_m values for tetrahydrololate and ATP was observed on the addition of NH_4^+.

Phosphate ions inhibit formyltetrahydrofolate synthetase from *C. cylindrosporum* in a complex fashion [14]. We found that phosphate inhibits also the enzyme from *C. thermoaceticum*. This inhibition is stronger in the presence of NH_4^+. Thus in assays with saturating conditions of formate, ATP and tetrahydrofolate the presence of 20 mM phosphate (Tris salt) lowers the V 20% in the absence of NH_4Cl but 60% in the presence of 50 mM NH_4Cl.

Apparent K_m values for the forward reaction as a function of temperature in the presence and absence of NH_4Cl

A change in the enzyme at 40–43 °C is indicated by changes in the apparent K_m values of all the substrates (Fig. 5). In the presence of 2 mM NH_4Cl, the K_m values for Mg-ATP and tetrahydrofolate increase linearly with temperature, with slope changes for both substrates at 40–43 °C. The formate K_m is independent of temperature below 40–43 °C in the presence of NH_4Cl but increases linearly above. In the absence of ammonium chloride, the K_m values for Mg-ATP and tetrahydrofolate increase linearly below 40–43 °C, and are independent of temperature above. The K_m for formate increases linearly with temperature with a slope change at 40–43 °C.

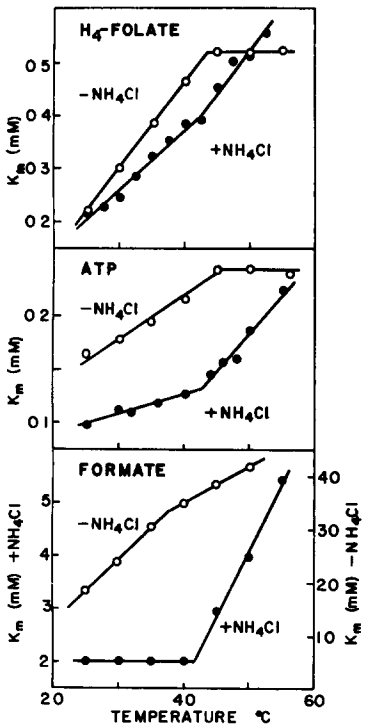

Fig. 5. Apparent K_m values of the substrates in the forward reaction as a function of temperature and the effect of NH_4Cl. For determinations of the apparent K_m values of ATP and tetrahydrofolate, the concentration of formate was 40 mM in the presence of NH_4Cl, and 100 mM in the absence. In the determination of ATP K_m values, the Mg:ATP ratio was maintained at 2:1. NH_4^+ when present was 2 mM.

Activation of the reverse reaction by NH_4^+

When the reverse reaction was measured in the routine way (except that NH_4Cl is excluded), the reaction was stimulated approximately 4-fold by the addition of 20 mM NH_4Cl. Lineweaver–Burke plots with phosphate and 10-formyltetrahydrofolate as variable substrates (Fig. 6A, B) indicate that NH_4^+ stimulates the reaction

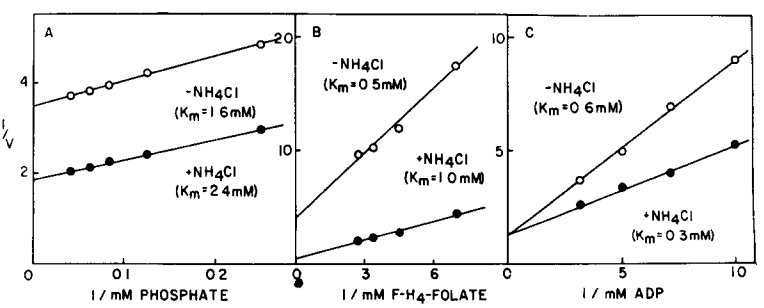

Fig. 6. Effect of NH_4Cl on the velocity of the reverse reaction. NH_4Cl when present was 20 mM. When used as a non-variable substrate, ADP was 1 mM, phosphate was 40 mM, and 10-formyltetrahydrofolate was 0.35 mM. 6 units (measured in forward direction) of enzyme was used in each sample.

by increasing the V and perhaps also by lowering their apparent K_m values. With ADP as the variable substrate, the effect seems to be only on the apparent K_m (Fig. 6C), which is lowered approximately 2-fold by the addition of 20 mM NH_4Cl.

Arrhenius plots

The synthetase from *C. thermoaceticum* exhibits a broken Arrhenius plot for the forward reaction both in the presence and absence of ammonium chloride (Fig. 7). The activation energy for the reaction is higher below 40–43 °C than above. In the presence of 2 mM NH_4Cl, the change in activation energy is small, decreasing from 10 400 to 8900 cal/mole as the temperature is increased. In the absence of NH_4Cl or KCl, this change is much larger, decreasing from 7150 to 500 cal/mole at 40–43 °C.

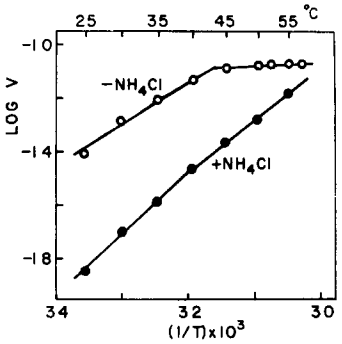

Fig. 7. Log of velocity of formyltetrahydrofolate synthetase as a function of the reciprocal of the absolute temperature. Assays were as described in Fig 2 except that the total volume was 1.0 ml. In the absence of NH_4Cl 0.1 unit of enzyme was used, while in the presence of 2 mM NH_4Cl, 0.033 unit of enzyme was used. Aliquots of 0.1 ml were taken at 1-min intervals and added to 1.0 ml of 0.29 M HCl to determine initial velocities.

Fluorescence emission as a function of temperature

The synthetase was excited at 278 nm in the presence and absence of NH_4Cl. The fluorescence emission maximum was approximately at 325 nm. The position of the emission maximum shifted from 325 to 328 nm with increasing temperature both in the presence and in the absence of NH_4^+. When fluorescence intensity (at the peak) was plotted as a function of temperature (Fig. 8), it was found to decrease linearly with increasing temperature in the absence of ammonium chloride. This fluorescence change was not reversible in the absence of NH_4^+ since the enzyme then is slowly denatured at temperatures greater than 50 °C. In the presence of 2.5 mM NH_4Cl, the fluorescence decreased linearly but with a slope change at 40–43 °C. This change was reversible as the temperature was decreased to approximately 26 °C.

Effect of temperature on ultraviolet absorbance

The absorbance (at 295 nm) of the synthetase was measured as a function of temperature from 25 to 50 °C (Fig. 9). A linear increase in absorbance up to approximately 40 °C occurred in the presence or absence of NH_4Cl. At 40 °C a slope change occurred and the change in absorbance was again linear until precipitation occurred (as indicated by a large increase in absorbance). The temperature at which precipita-

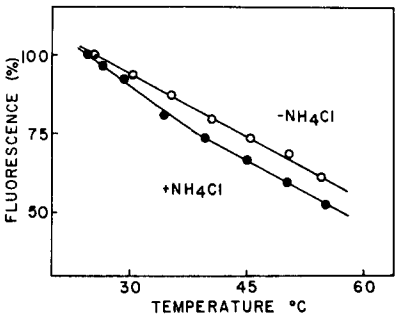

Fig. 8. Fluorescence intensity of formyltetrahydrofolate synthetase as a function of temperature. Synthetase was dialyzed versus 0.1 M maleate (Tris), pH 8, and 0.92 mg of the enzyme was placed in a solution containing 0.1 M maleate (Tris), pH 8, and when present 2 5 mM NH_4Cl in a total volume of 2 ml. The sample was excited at 278 nm and fluorescence intensity at the emission peak (325 nm) was recorded. The time required to obtain the measurements for one curve was approximately 2 h.

tion occurred was higher in the presence of 2 mM NH_4Cl than in its absence and 2 mM NaCl had no effect. The change in absorbance observed was very small, and to observe it, protein concentrations in the range of 2.5–3 mg/ml were necessary. With some preparations, the non-ammonium sample did not show a break at 40 °C. We have not established the cause as yet, but this may be related to the length of time the enzyme has been in the absence of NH_4^+.

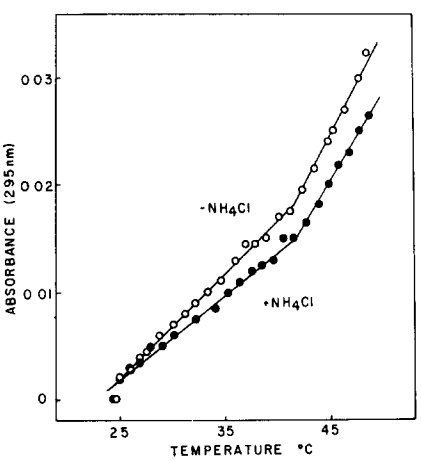

Fig. 9. Effect of temperature on ultraviolet absorbance of formyltetrahydrofolate synthetase. The enzyme used was dialyzed versus 0.05 M Tris–HCl, pH 8. The sample cuvette contained 3 mg protein per ml. 50 mM Tris–HCl, pH 8, and when present, 2 mM NH_4Cl .Initial absorbance at 25 °C and 295 nm was 0.45. The reference cuvette contained an identical sample minus enzyme. Absorption measurements were performed using a double beam Beckman Acta CV recording spectrophotometer.

Optical rotatory dispersion measurements

ORD measurements were made on a Cary 60 spectropolarimeter. The enzyme, measured in 0.5 mM Tris–HCl buffer, pH 7.5, exhibited a prominent Cotton effect in the ultraviolet region with a trough at approximately 230 nm. The profile was

unchanged by the addition of NH_4Cl at 26 °C. Likewise the profile did not change on increasing the temperature from 24 to 56 °C, except that a small increase was noticed in the depth of the trough at 230 nm.

DISCUSSION

Formyltetrahydrofolate synthetase isolated from the obligative thermophile *C. thermoaceticum* is more heat stable and has a higher temperature optimum than the same enzymes from other *Clostridia* [5]. The enzyme from *C. cylindrosporum* is enzymatically active only as a tetramer. The presence of monovalent cations (NH_4^+ or K^+) is required to maintain the tetrameric structure and monomers are obtained by removing these ions through dialysis [15]. The removal of NH_4^+ or K^+ from the *C. thermoaceticum* enzyme does not lead to the formation of monomers even at a temperature as high as 50 °C or slightly above [5]. However, as shown in a previous [5] and in this paper NH_4^+ or K^+ increase the thermostability of the *C. thermoaceticum* enzyme. Furthermore they affect the kinetic properties in a way similar to that of the *C. cylindrosporum* enzyme. The most pronounced effect is the 10-fold lowering of the K_m for formate by NH_4^+ from 50 mM to 5 mM with both enzymes [16]. The fact that the *C. thermoaceticum* enzyme has a stable tetrameric structure at high temperatures without the presence of NH_4^+ or K^+, indicates that there is a much stronger interaction between the subunits of this enzyme compared with the *C. cylindrosporum* enzyme. Perhaps the higher thermostability of the *C. thermoaceticum* enzyme is a result of the stronger interaction between the subunits.

The interaction between the monomers in forming the tetrameric structure may well be hydrophobic. Thus Welch et al. [10] have presented evidence with the *C. cylindrosporum* enzyme that tryptophan, tyrosine and phenylalanine residues become buried in hydrophobic regions when the subunits reassociate. We have previously presented fluorescence data taken in the presence of potassium maleate which indicate that with the *C. thermoaceticum* enzyme a shift of tryptophan residues to a more hydrophobic environment occurs when going from lower to higher temperatures [9]. The presence of monovalent cations may enhance the hydrophobic interaction for instance by neutralizing negative charges. It has been suggested that hydrophobic interactions between amino acid residues may stabilize proteins at high temperatures [17].

The results presented in Figs 8 and 9 show that formyltetrahydrofolate synthetase undergoes a transition at approximately 40–43 °C which affects the apparent K_m values of the substrates for the enzyme and lowers the activation energy for the reaction. The non-linear Arrhenius plots can be a result of kinetic or physical changes in the enzyme as discussed by Han [18]. Phase changes in the solvent could also produce discontinuities in Arrhenius and K_m plots [19]. Present data do not however indicate that phase changes in water could account for the observed breaks. Breaks in Arrhenius plots and K_m plots have been reported for many types of enzymes over a wide range of temperatures from 0 to 55 °C and such breaks are not limited to thermophilic enzymes [21–29]. Thus it seems unlikely that such breaks are a result of phase changes in the solvent.

The fluorescence change indicated in Fig. 8 and the absorbance change shown in Fig. 9 suggest that the enzyme does undergo a physical change, albeit very small.

Both figures show breaks at approximately 40–43 °C, the temperature at which breaks occur when the kinetic parameters are plotted. That a physical change has been observed at approximately the same temperature as the kinetic changes, which occur in the presence as well as in the absence of ammonium ions, indicates that the kinetic changes may be the result of a temperature dependent conformational change in the protein.

Suelter [20] has reviewed the evidence that monovalent cations may play an essential role in the reaction mechanism of certain enzymes. The mechanism of the formyltetrahydrofolate synthetase from *C. cylindrosporum* has been determined and was found to be random terter [30]. Since NH_4^+ does not effect the V of the reaction and is not required for activity it has been suggested that it does not play an essential part of the reaction mechanism [15]. We agree with this suggestion and believe it is valid also for the enzyme from *C. thermoaceticum*. However, we do not know the rate limiting step of the reaction and the participation of NH_4^+ in the catalytic process can not be excluded.

It appears that the involvement of NH_4^+ may be primarily in a structural role. They make the enzyme more stable to heat inactivation (Fig. 1), less reactive with *p*-hydroxymercuribenzoate (Fig. 2) and affect although slightly the fluorescence (Fig. 8) and the absorbance (Fig. 9). Based on these observations together with the results discussed earlier regarding effects of temperature and ammonium ions on K_m values and on activation energies we would like to suggest that the enzyme from *C. thermoaceticum* exists in several conformations as outlined in Fig. 10. The A and B conformation exist in the absence of NH_4^+ and conformation A is predominant below 40 °C

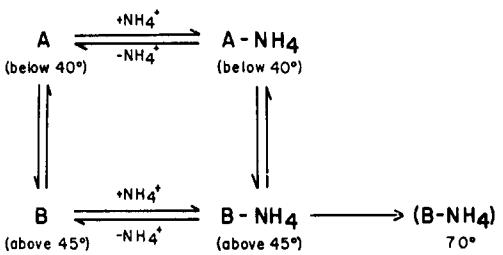

Fig. 10. Conformational forms of formyltetrahydrofolate synthetase from *C. thermoaceticum*.

while conformation B exists above 45 °C. Addition of NH_4^+ (or perhaps K^+) to either the A or B enzyme converts these species to A-NH_4 or B-NH_4 enzymes. The conversion between A, B, A-NH_4 and B-NH_4 conformational forms is freely reversible. It is possible that a fifth conformational form exists, designated (B-NH_4) 70 °C. This species is seen when the enzyme is heated for a period at 70 °C and the transition may be irreversible.

ACKNOWLEDGEMENTS

The authors wish to thank Ms Chris Neurauter for preparation of the purified formyltetrahydrofolate synthetase used in these experiments.

This investigation was supported by Grant AM 12913 from the National Institutes of Health and GB 13031 from the National Science Foundation.

REFERENCES

1 Shoaf, W. T., Ljungdahl, L. G., Brewer, J. M. and McRorie, R. A. (1973) Annual Meeting of American Society of Microbiology, Abstr. Annu. Meeting P156
2 Blakley, R. L. (1969) The Biochemistry of Folic Acid and Related Pteridines, John Wiley and Sons, Inc., New York
3 Rabinowitz, J. C. and Pricer, W. E. (1962) J. Biol Chem. 237, 2898–2902
4 Curthoys, N. P. and Rabinowitz, J. C. (1971) J. Biol. Chem 246, 6942–6952
5 Ljungdahl, L. G., Brewer, J. M , Neece, S. H and Eairwell, T (1970) J Biol. Chem. 245, 4791–4797
6 O'Brien, W. E. and Ljungdahl, L G. (1972) J. Bacteriol. 109, 626–632
7 Ljungdahl, L. G. and Wood H. G. (1969) Annu. Rev. Microbiol. 23, 515–538
8 Andreesen, J. R , Schaupp, A., Neurauter, C., Brown, A and Ljungdahl, L. G. (1973) J Bacteriol. 114, 743–757
9 Brewer, J. M., Ljungdahl, L. G., Spencer, T. E. and Neece, S H. (1970) J Biol. Chem. 245, 4798–4803
10 Welch, W. H., Buttlaire, D. H , Hersh, R. T and Himes, R. H (1971) Biochim. Biophys. Acta 236, 599–611
11 Welch, W. H., Irwin, C L. and Himes, R. H. (1968) Biochem. Biophys. Res. Commun. 30, 255–261
12 Scott, J. M. and Rabinowitz, J. C (1967) Biochem. Biophys. Res Commun 29, 418–423
13 Rowe, P. B. (1971) Methods Enzymol. 18B, 733–735
14 Joyce, B. K. and Himes, R. H. (1966) J Biol. Chem. 241, 5725–5731
15 Curthoys, N. P., D'Ari Strauss, L. and Rabinowitz, J C (1972) Biochemistry 11, 345–349
16 Himes, R. H. and Wilder, T. (1965) Biochim. Biophys. Acta 99, 464–475
17 Brandts, J. F. (1967) Thermobiology (Rose, A H., ed.), Academic Press, New York
18 Han, M. H. (1972) J. Theor. Biol. 35, 543–568
19 Dixon, M. and Webb, E C. (1965) in Enzymes, 2nd edn, pp. 158 ff, Academic Press, New York
20 Suelter, C. H. (1970) Science 168, 789–795
21 Sizer, I. W. and Josephson, E S. (1942) Food Res. 7, 201
22 Massey, V., Curti, B. and Ganther, H. (1966) J. Biol. Chem 241, 2347–2357
23 Massey, V. (1953) Biochem J. 53, 72–79
24 Lehrer, G. M. and Barker, R (1970) Biochemistry 9, 1533–1539
25 Himes, R H. and Wilder, T. (1968) Arch. Biochem. Biophys. 124, 230–237
26 Orengo, A. and Saunders, G F. (1972) Biochemistry 11, 1761–1767
27 O'Brien, W. E., Brewer, J. M. and Ljungdahl, L. G. (1973) J. Biol. Chem. 248, 403–408
28 Sugimoto, S. and Nosoh, Y. (1971) Biochim. Biophys Acta 235, 210–221
29 Hachimori, A., Marumatsu, N and Nosoh, Y. (1970) Biochim. Biophys. Acta 206, 426–437
30 Joyce, B. K. and Himes, R. K (1966) J. Biol. Chem. 241, 5716–5724