OBSERVATIONS ON THE MONOVALENT CATION REQUIREMENTS OF FORMYLTETRAHYDROFOLATE SYNTHETASE

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The requirement of formyltetrahydrofolate synthetase (E.C.6.3.4.3.) for monovalent cations (Whitely and Huennekens, 1962; Himes and Wilder, 1965) has been correlated with the ability of the cations to prevent the dissociation of the native enzyme into subunits (Scott and Rabinowitz, 1967). In our laboratory we have also shown that dissociation of the enzyme occurs in the absence of certain cations and leads to inactivation of the enzyme. The data presented in this report show that the inactivation process is retarded by low concentrations of NH_4^+, comparatively higher concentrations of multivalent anions, and the substrates for the reaction. In addition, they show that the enzyme, stabilized in the absence of a monovalent cation, has a Km for formate which is 10 times as large as that obtained with the monovalent cation-stabilized enzyme. The Km values for MgATP and tetrahydrofolate, however, are the same for both forms of the enzyme.

Experimental - Formyltetrahydrofolate synthetase was prepared and assayed as previously described (Himes and Rabinowitz, 1962a). Prior to the experiments the crystalline enzyme (in 50% ammonium sulfate, 0.05 M potassium maleate, and 0.1 M 2-mercaptoethanol) was collected by centrifugation dissolved in 0.5 ml of the appropriate buffer and passed through a column of Sephadex G-25 at 5°. Passage of the enzyme

through the column in 0.1 M tris·HCl led to variable yields of the activity. To avoid these losses of activity the buffers were supplemented with either 5 x 10^{-4} M NH_4^+ or 0.1 M sulfate. Prior to the inactivation studies, the enzyme was diluted approximately 100 fold.

Results - In a previous report Himes and Wilder (1965) showed that at 37° formyltetrahydrofolate synthetase from Clostridium cylindrosporum has an absolute requirement for a monovalent cation. More recently we have observed that when the reaction is performed at 20° or 10° the activity is stimulated only 2 to 3 fold by NH_4^+. In addition, if the concentration of the monovalent cation is too low, the velocity at 37° is constant for only 3-4 minutes after which it rapidly falls to zero. On the other hand, at 10° and 20° the velocity is constant for at least 20 minutes even in the absence of an active monovalent cation. Thus, in the presence of the substrates but in the absence of a sufficient concentration of an activating cation the enzyme at 37° is not stable over the time period normally used in kinetic studies (10 minutes).

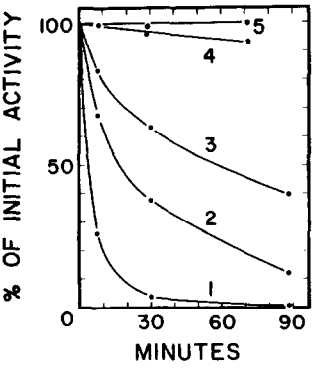

Fig. 1. Effect of NH_4^+ concentration and the substrates on enzyme inactivation. After passing through a column of Sephadex G-25 in 0.1 M tris·HCl which contained 0.5 mM NH_4Cl the enzyme was diluted 100 fold in 1 ml of 0.1 M tris·HCl containing the following final concentrations of NH_4^+: 1, 0.015 mM; 2, 0.05 mM; 3, 0.1 mM; 4, 1.0 mM; 5, 2.0 mM. Curve 5 also represents the data obtained when the NH_4^+ concentration was 0.005 mM and 5 mM ATP, 10 mM Mg^{++}, 40 mM formate, 2 mM ± tetrahydrofolate and 0.2 M 2-mercaptoethanol were present. Incubation was at 20°. At the times indicated 20 ul of sample was removed and assayed at 37° (Himes and Rabinowitz 1962a).

If both cation and substrates are missing the enzyme is quite unstable even at the lower temperatures. The rapid loss of activity which occurs under the latter conditions is retarded by NH_4^+ or a mixture of the substrates of the reation (Fig. 1). The specificity of the cation protection is shown in Table I.

Table I. Effect of Monovalent Cations on Inactivation in Tris·HCl

Cation	Percent Loss of Enzyme Activity	
	8 min	41 min
None	78	98
Lithium	84	99
Sodium	35	91
Cesium	26	79
Potassium	17	49
Rubidium	12	39
Ammonium	0	0
Methylammonium[a]	1	15
Di-, tri-, and tetramethyl-ammonium[a]	63	98

The enzyme (1.3×10^{-8} M) was incubated at 20° in 1 ml of 0.1 M tris·HCl, pH 7.7, which contained 1 mM sulfate and 1 mM of each cation added as the chloride salt.

a. In the experiments with the methyl ammonium derivatives the times of incubation were 6 and 39 minutes.

In agreement with previous kinetic studies (Himes and Wilder, 1965) we find that NH_4^+ is the most effective cation in protecting against inactivation (Table I). Increasing the tris·HCl concentration to 0.4 M did not improve the effectiveness of this ion. The protective effect shown by NH_4^+ is decreased by substitution of the hydrogens with methyl groups.

Methylammonium chloride is effective, whereas di, tri, and tetramethylammonium chlorides are not (Table I). In addition to tris·HCl the enzyme is quite unstable in triethanolamine·HCl and N-ethylmorpholine·HCl.

The chemical nature of the anion in the buffer is very important since, in tris buffer, various multivalent anions retard the rate of inactivation. For example, in 0.1 M solutions of maleate, sulfate, fumarate or suberate the enzyme lost no activity after 1 hour at 20° whereas the control which contained chloride as the anion decreased in activity by 72%. However, 100 mM concentrations of the anions are required to effect stability for 1 hour whereas 1 mM NH_4^+ is sufficient.

The enzyme was found to be stable in the absence of NH_4^+ when incubated in a mixture of the tris salts of the substrates (see Fig. 1). In the presence of 0.1 M tris·HCl, pH 7.7; 5 mM ATP; 10 mM Mg^{++}; 40 mM formate; 2 mM $\pm$ tetrahydrofolate; and 0.2 M 2-mercaptoethanol; and at 20°, the enzyme (1.5×10^{-8} M) was stable for four hours. Each substrate contributed to the stability but there appeared to be little specificity for the substrates. For example, 1 mM GTP and CTP were as effective as 1 mM ATP in retarding inactivation even though GTP and CTP are not effective substrates or inhibitors (Himes and Rabinowitz, 1962b). The protective ability of the substrate mixture may be related to the protection obtained with anions.

Though the enzyme is stable at 20° for several hours in the presence of the substrates the activity at this temperature is stimulated 2 to 3 fold by NH_4^+. This stimulation was investigated further by determining the kinetic constants at 20° in the presence of, and in the absence of, NH_4^+. The results are presented in Table II.

The most significant change is the large increase in the Km of formate in the absence of monovalent cation. This change would explain the low V values obtained in the absence of NH_4^+ when MgATP or tetrahydrofolate were the variable substrates. Since the Km of formate under these

Table II. Effect of NH_4^+ on the Kinetic Constants of the Reaction

Variable Substrate	NH_4^+	Km,mM	Relative V
MgATP	+	0.16	1.0
	-	0.17	.37
± tetrahydrofolate	+	0.42	1.0
	-	0.58	.42
formate	+	5.3	1.0
	-	50	1.0

The constants were determined at 20° in 0.1 M tris·HCl, pH 8.0, and 0.2 M 2-mercaptoethanol. Except when they were being varied the concentrations of the substrates were; $MgCl_2$, 10 mM; ATP, 5 mM; ± tetrahydrofolate, 2 mM; and formate, 40 mM. When present the NH_4^+ concentration was 50 mM.

conditions is 50 mM the formate concentration was not saturating in these experiments. When formate was the variable substrate the V values in the presence and absence of NH_4^+ were equal.

Because of the different kinetic properties the enzyme exhibited in the presence and absence of $NH4^+$ we determined the sedimentation constant of the protein under the same conditions used for the kinetic experiments (except for enzyme concentration). The S_{20} in the presence of the substrates was 8.2 and in the presence of the substrates and NH_4^+ was 8.1.

Discussion - Scott and Rabinowitz (1967) have shown that formyltetrahydrofolate synthetase is a tetramer and that in the absence of an active monovalent cation it dissociates into enzymically inactive subunits. We have shown at a concentration one thousandth of that used by the latter authors that the inactivation process is very rapid and can be retarded by certain monovalent cations. NH_4^+ is by far the most effective cation in stabilizing the enzyme. The low NH_4^+ concentrations required for stabilization rule out ionic strength effects. Stabilization is also afforded by certain multivalent anions at higher concentrations

and by a combination of the substrates of the reaction.

Under conditions where the enzyme is completely stable (10 minutes at 20° in the presence of the substrates) NH_4^+ causes an apparent 2 to 3 fold stimulation of enzymic activity under normal assay conditions. Kinetic data show that the stimulation is a result of a drastic change in the Km of formate. The formate concentration used in the normal enzyme assay is sufficient to saturate the enzyme when NH_4^+ is present but approximately half saturates the enzyme in the absence of NH_4^+. When the formate concentration is infinite there is no stimulation by NH_4^+. These data suggest that two active forms of the enzyme exist in equilibrium. The form with the lower Km for formate (form A) predominates in the presence of NH_4^+ and the substrates. Form B predominates in the presence of the substrates alone (and perhaps in the presence of multivalent anions). Since NH_4^+ causes no change in the sedimentation coefficient of the protein determined in the presence of the substrates we conclude that there is a conformational difference in the two forms which is sufficient to cause a change in the affinity for formate but not in the sedimentation coefficient. The dissociation of the enzyme occurs in the absence of any stabilizing factor (monovalent cation, multivalent anion, or substrates). Whether several steps are involved in subunit formation with form B as an intermediate or whether subunits can arise directly from both forms A and B is not known.

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