L(−)-10-Formyltetrahydrofolate Is the Cofactor for Glycinamide Ribonucleotide Transformylase from Chicken Liver†

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ABSTRACT: It is shown that L(−)-10-formyltetrahydrofolate serves as the cofactor for glycinamide ribonucleotide transformylase from chicken liver. The utilization of L(−)-10-formyl-H_4folate was not previously recognized, because L-(+)-10-formyl-H_4folate is an excellent competitive inhibitor of the enzyme, $K_i = 0.75 \pm 0.07$ μM, and historically the transformylase assay was carried out with a mixture of diastereomers. The results are discussed in relation to the utilization of L(+)-5,10-methenyl-H_4folate.

Buchanan and co-workers first characterized the GAR TFase[1] reaction in a crude preparation from chicken liver (Hartman & Buchanan, 1959). They showed that the cofactor is 5,10-methenyl-H_4folate[1] and that the overall reaction is

GAR + 5,10-methenyl-H_4folate → FGAR + H_4folate

We have recently shown that a second enzyme, the trifunctional protein, is required in addition to GAR TFase to catalyze the reaction as written (Smith et al., 1980). The trifunctional protein apparently binds the 5,10-methenyl-H_4folate from solution and then "furnishes" a form of the cofactor to the GAR TFase binding site. We have also shown that a number of 10-formyl analogues (such as 10-formyl-5,8-deazafolate) can bypass this requirement and bind directly to GAR TFase to formylate GAR in the presence or absence of the trifunctional protein (Smith et al., 1981). These results suggested to us that in the natural reaction the active form of the cofactor is not 5,10-methenyl-H_4folate but rather 10-formyl-H_4folate and led us to reinvestigate the direct utilization of 10-formyl-H_4folate as a cofactor.

The results of the present study show that the L(−)-10-formyl-H_4folate (the hydrolysis product of L(+)-5,10-methenyl-H_4folate) is the true cofactor for GAR TFase. Severe inhibition by L(+)-10-formyl-H_4folate in chemical preparations of L(±)-10-formyl-H_4folate had previously masked this activity. These results are in agreement with the report that *Escherichia coli* GAR TFase also utilizes 10-formyl-H_4folate as its cofactor (Dev & Harvey, 1978)

Experimental Procedures

Materials. GAR TFase, the trifunctional protein, L(±)-5,10-methenyl-H_4folate, and (α,β)GAR were prepared as described previously (Smith et al., 1980). H_2folate was prepared according to the procedure of Blakley (1960), and L-(−)-H_4folate was produced from the H_2folate with H_2folate reductase (Mathews & Huennekens, 1960) and purified on DEAE-cellulose eluting with a 0.2–0.7 M NH_4HCO_3, pH 8.0, linear gradient (Curthoys et al., 1972). L(+)-5,10-Methenyl-H_4folate was synthesized from L(−)-H_4folate according to the method of Rowe (1968). L(−)-5,10-Methenyl-H_4folate was a generous gift of the Southern Research Institute. Hydrolysis of (+)- and (−)-5,10-methenyl-H_4folate gave (−)- and (+)-10-formyl-H_4folate, respectively.

Methods. All solutions, except enzymes which were always added in small amounts, were thoroughly degassed by alternately evacuating and saturating with argon with at least eight exchanges. All reactions were performed in argon-flushed, serum cap sealed tubes or cuvettes. Additions and transfers were made through syringes.

Buffer-catalyzed hydrolysis of 5,10-methenyl-H_4folate to 10-formyl-H_4folate was performed at room temperature in 40 mM potassium phosphate and 0.1 mM EDTA, pH 6.8. The reaction was allowed to proceed in the dark for 2 h, at which time it was judged complete by the product UV spectrum (Rabinowitz, 1963). The solution was used for that day's work, after which recyclization to 5,10-methenyl-H_4folate in 0.1 M HCl indicated very little decomposition.

GAR TFase assays were performed at pH 6.8 in 50 mM maleate buffer containing 0.1 mM EDTA, 0.259 mM (α,β)-GAR, and varying amounts of 5,10-methenyl- or 10-formyl-H_4folate. The Bratton and Marshall assay for diazotizable amine was performed as previously described (Smith et al., 1980). Spectrophotometric assays were performed in a 1-mL reaction volume in a serum cap sealed cuvette. Absorbance changes were monitored at either 298 or 312 nm in a Gilford 240 spectrophotometer equipped with constant temperature and automatic recording accessories. Assay solutions were preincubated for 10 min at 35 °C in the absence of cofactor, and the reaction was initiated by adding the latter in a small volume. No difference was observed in the 10-formyl-H_4folate activity with GAR TFase whether enzyme or cofactor was used to start the reaction.

The Cleland programs for linear competitive and noncompetitive inhibition were used to analyze inhibition data (Cleland, 1967).

Results

The Bratton–Marshall diazotizable amine assay, typically used with GAR TFase, is a point assay requiring an acid quench and a four-step color development. For determination of the entire reaction time course, it is cumbersome and time consuming; thus, a spectrophotometric assay was sought.

The spectral change for the buffer-catalyzed hydrolysis of 5,10-methenyl-H_4folate to 10-formyl-H_4folate in 40 mM potassium phosphate, pH 6.8, is shown in Figure 1. The reaction shows an isosbestic point at 312 nm. At this wavelength, the difference in molar absorptivity between H_4folate and either

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[1] Abbreviations used: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; GAR, glycinamide ribonucleotide; H_4folate, tetrahydrofolate; TFase, transformylase; FGAR, formylglycinamide ribonucleotide; trifunctional protein, 5,10-methenyl-, 5,10-methylene-, 10-formyl-H_4folate synthetase (combined). The (+), (−), or (±) notation is the conventional optical rotation designation for a given cofactor species.

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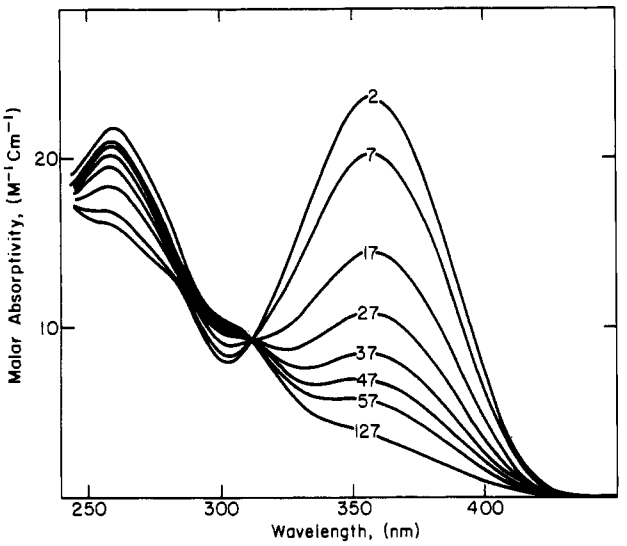


FIGURE 1: Buffer-catalyzed hydrolysis of 5,10-methenyl-H_4folate. The reaction was performed at 25 °C in 40 mM potassium phosphate buffer, pH 6.8. Times recorded on the spectra are in minutes.

Table I: Utilization of L(±)-10-Formyl- and L(±)-5,10-Methenyl-H_4folate by GAR TFase

cofactor	[cofactor] (μM)	$v_0\Delta A_{312/min}$[a] without trifunctional protein	with trifunctional protein
L(±)-10-formyl-H_4folate	50	0.0030[b]	0.0030
L(±)-10-formyl-H_4folate	25	0.0027[b]	0.0029
L(±)-5,10-methenyl-H_4folate	50	0.0045[c]	0.019
L(±)-5,10-methenyl-H_4folate	25	0.0032[c]	0.0165

[a] The enzymes were preincubated in the absence of cofactor for 10 min. For the GAR TFase enzyme, 2.5 μL of a solution of a 0.712 mg/mL GAR TFase and 0.054 mg/mL trifunctional protein was used. For the trifunctional protein, 5.0 μL of 0.2 mg/mL solution was used. [b] Adding trifunctional protein to the running assay had no effect. [c] Adding trifunctional protein to the running assay gave the expected activation within the mixing time.

5,10-methenyl-H_4folate or 10-formyl-H_4folate is 12000 M^{-1} cm^{-1}. Thus, the formylation of GAR with 5,10-methenyl-H_4folate can be followed with no interference from the 5,10-methenyl-H_4folate hydrolysis reaction. Similarly, the formylation from 10-formyl-H_4folate alone can be followed at 298 nm, as shown for AICAR TFase by Black et al. (1978): $\Delta\epsilon_{298}$ = 19700 M^{-1} cm^{-1}. Using these spectral changes and the Bratton–Marshall technique, we have reinvestigated the cofactor requirement of GAR TFase from chicken liver.

It has previously been assumed that any GAR TFase activity found with 10-formyl-H_4folate as the cofactor was due to residual 5,10-methenyl-H_4folate in solution (Caperelli et al., 1980). However, we have shown that the GAR TFase activity with 5,10-methenyl-H_4folate requires the presence of the trifunctional protein while the activity with 10-formylfolate analogues does not (Smith et al., 1980, 1981); thus, if 10-formyl-H_4folate utilization is the same in the presence or absence of the trifunctional protein, the activity cannot result from residual 5,10-methenyl-H_4folate. The results of four assays with L(±)-10-formyl-H_4folate compared with similar assays with L(±)-5,10-methenyl-H_4folate are shown in Table I. It can be seen that there is identical utilization of the 10-formyl-H_4folate with or without the trifunctional protein

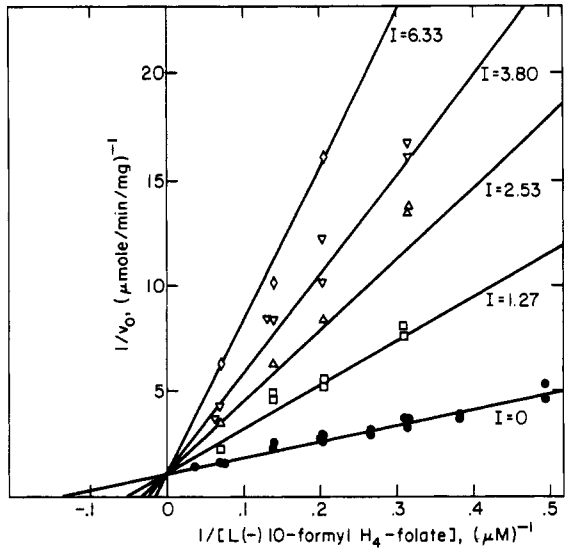


FIGURE 2: Double-reciprocal plot of the utilization of L(−)-10-formyl-H_4folate by GAR TFase and its inhibition by L(+)-10-formyl-H_4folate. Assay performed at 35 °C in 50 mM maleate, 0.1 mM EDTA, 0.259 mM (α,β)GAR, and 2.0 μg of complex. ΔA_{312} was recorded. Inhibitor concentrations are μM.

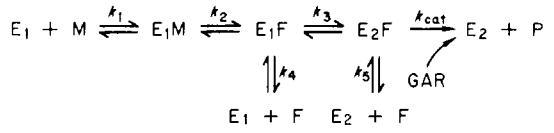
whereas efficient use of the 5,10-methenyl-H_4folate as a cofactor shows a requirement for the trifunctional protein. The V_{max} for L(±)-10-formyl-H_4folate relative to L(±)-5,10-methenyl-H_4folate is 14% (0.080 compared to 0.59 μmol min^{-1} mg^{-1} for a typical enzyme complex preparation). It could be argued that the failure of the trifunctional protein to increase the utilization of the L(±)-10-formyl-H_4folate by GAR TFase is due to slow activation. However, no increase over the initial velocity with the 10-formyl species is observed during the assay period (up to 15 min) whereas activation of the 5,10-methenyl-H_4folate utilization occurs within the 15–20 s required for adding and mixing the last reagent (cofactor or trifunctional protein).

The assay was then repeated with L(−)-10-formyl-H_4folate, and it was found that the rate was identical with the rate with L(+)-5,10-methenyl-H_4folate (V_{max} L(+)-methenyl-H_4folate = 0.91 ± 0.05 μmol min^{-1} mg^{-1} and L(−)-formyl-H_4folate = 0.95 ± 0.07 μmol min^{-1} mg^{-1} for the same complex used above). Again the trifunctional protein had no effect on the rate with 10-formyl-H_4folate but was required for L(+)-5,10-methenyl-H_4folate utilization, activating within the assay dead time (15–20 s). The reason for the large difference between the utilization of L(±)-10-formyl-H_4folate and L-(−)-10-formyl-H_4folate is shown in Figure 2 where the competitive inhibition due to L(+)-10-formyl-H_4folate is displayed. It can be seen that the wrong diastereomer of 10-formyl-H_4folate severely inhibits GAR TFase, and from this plot, a K_i of 0.75 ± 0.07 μM for L(+)-10-formyl-H_4folate was calculated, compared to a K_m of 6.76 ± 0.65 μM for L(−)-10-formyl-H_4folate.

An attempt was made to fit the data in Figure 2 to a noncompetitive scheme (Cleland, 1967). However, the K_{II} (intercept derived or noncompetitive K_I) calculated is negative, −3.74 ±1.5 μM. Thus, a noncompetitive model does not fit, and the competitive nature of the inhibition by L(+)-10-formyl-H_4folate is substantiated.

The K_m for L(−)-10-formyl-H_4folate in the absence of the trifunctional protein is 5.94 ± 0.77 μM. Thus, the trifunctional protein affects neither the K_m nor the V_{max} of L(−)-10-formyl-H_4folate. The K_m for L(+)-5,10-methenyl-H_4folate was recalculated by employing this assay and gave K_m = 5.76 ± 0.60 μM.

Scheme I



Discussion

The results show that L(−)-10-formyl-H_4folate is the active cofactor for GAR TFase in the absence of the trifunctional protein. This was not observed previously, because L(+)-10-formyl-H_4folate is surprisingly an excellent competitive inhibitor (K_i = 0.75 μM); thus, the presence of this diastereomer of the cofactor in L(±)-10-formyl-H_4folate formed by chemical reduction caused the reduced rate found with L(±)-10-formyl-H_4folate compared to L(±)-5,10-methenyl-H_4folate. (The latter is selectively hydrolyzed to the L(−)-10-formyl-H_4folate by the cyclohydrolase activity present in earlier preparations of GAR TFase.) Consequently the slow rate of (±) cofactor utilization in a solution at pH 6.8 where the cofactor concentrations are equilibrated (10-formyl-H_4folate:5,10-methenyl-H_4folate in the ratio 1:0.15; Poe & Benkovic, 1980) was presumed to originate from the residual 5,10-methenyl-H_4folate in the solution.

The activity of GAR TFase in the presence of the trifunctional protein when assayed with L(±)-5,10-methenyl-H_4folate is lower than that with L(+)-5,10-methenyl-H_4folate (0.59 compared to 0.91 μmol min^{-1} mg^{-1}). However, it cannot be determined whether this is due to slight inhibition from the L(−)-5,10-methenyl-H_4folate or from the small amount of L(+)-10-formyl-H_4folate which is certain to arise from buffer-catalyzed hydrolysis.

The probable role of the trifunctional protein in the GAR TFase–trifunctional protein mixture is outlined in Scheme I, where E_1 ≡ cyclohydrolase activity of the trifunctional protein, E_2 ≡ GAR TFase, M ≡ 5,10-methenyl-H_4folate, F ≡ 10-formyl-H_4folate, P ≡ FGAR, and k_i's are net rate constants. The evidence for postulating this scheme is that 10-formyl-H_4folate is used by GAR TFase in the absence of the trifunctional protein whereas 5,10-methenyl-H_4folate use requires *active* trifunctional protein.

In one limit, solutions M and F are at chemical equilibrium, and 10-formyl-H_4folate from solution is the true cofactor. This is met by the condition k_i (i = 1, 2, and 4) $\gg k_3$. In the present preparation, the cyclohydrolase has a specific activity of 2.80 units/mg at 15 μM L(+)-5,10-methenyl-H_4folate. Thus, the 2 μg of complex used in the assays would produce 1.9 μM 10-formyl-H_4folate in the 20-s mixing time of the assay. Consequently the GAR TFase velocity should have a pronounced lag while the 10-formyl-H_4folate concentration builds toward 13 μM. Further, the apparent K_m calculated from the initial velocity data with 5,10-methenyl-H_4folate should be greater than that found for 10-formyl-H_4folate since the initial 10-formyl-H_4folate concentration is less than the 15 μM L-(+)-5,10-methenyl-H_4folate added. Neither of these criteria is fulfilled since the initial velocity at all 5,10-methenyl-H_4-folate concentrations is constant and K_m[L(+)-5,10-methenyl-H_4folate] = K_m[L(−)-10-formyl-H_4folate]. Thus, the role of the trifunctional protein is not simply to generate active cofactor. Additional evidence inferring direct channeling between the two proteins was furnished by the demonstration of GAR TFase and trifunctional protein cross-linking, of their association in the presence of GAR, and of the increased rate of FGAR synthesis commencing with formate compared to that with 5,10-methenyl-H_4folate (Smith et al., 1980).

The relative values of k_i (i = 1–5) will determine the ratio of cofactor channeling to that dissociating to solution and influence K_m. However, the similarity in K_m values for L-(+)-5,10-methenyl-H_4folate and L(−)-10-formyl-H_4folate does not readily follow from Scheme I without compensation within the values of k_i since two differing routes are utilized to saturate E_2F.

With the results presented here for GAR TFase and those of Hartman & Buchanan (1959) for AICAR TFase, it can be seen that both of the transformylases involved in purine biosynthesis employ L(−)-10-formyl-H_4folate as the common cofactor. The advantage of a direct formyl transfer agent rather than an amidinium donor in formylation may reside partially in the simplification afforded by controlling at the active site the bidirectional partitioning of a putative tetrahedral intermediate derived from the 10-formyl cofactor rather than the tridirectional routes open to a transient orthoamide species generated from the 5,10-methenyl cofactor.

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