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Direct Transfer of One-Carbon Units in the Transformylations of de Novo Purine Biosynthesis†

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ABSTRACT: It is shown that the transfer of formyl units in the de novo purine biosynthetic pathway as catalyzed by glycinamide ribonucleotide (GAR) transformylase and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase probably proceeds through a direct displacement mechanism involving only formyl donor (10-CHO-H_4folate) and formyl acceptor (GAR or AICAR). The inability to observe enzyme-catalyzed solvent oxygen incorporation or uncoupling by hydroxylamine of 1:1 stoichiometry between formylated acceptor [formylglycinamide ribonucleotide or 5-(formylamino)imidazole-4-carboxamide ribonucleotide] and deformylated donor implies the absence of an amidine intermediate and suggests that either a formylated enzyme-bound intermediate is not formed or such an intermediate is not accessible to hydroxylamine.

AICAR[1] transformylase and GAR transformylase are the two reduced folate requiring transformylases in de novo purine biosynthesis. They catalyze the formylation of AICAR and GAR, respectively, by using 10-formyl-H_4folate as the cofactor (Hartman & Buchanan, 1959; Smith et al., 1981a) to produce FAICAR and FGAR.

The mechanism of formyl transfer may involve the nucleophilic attack by the amino group of the acceptor (GAR or AICAR) to lead to products in one step—direct transfer. Alternatively, a nucleophilic function on the enzyme could attack the formyl donor and result in a covalently bound one-carbon unit at the formate level of oxidation—indirect transfer. In the latter case one may imagine two routes through either (1) a formyl enzyme or (2) an amidine composed of H_4folate and the transformylase that was formed by dehydration of a putative tetrahedral intermediate. Attack

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[1] Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; GAR, glycinamide ribonucleotide; FAICAR, 5-(formylamino)imidazole-4-carboxamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; TFase, transformylase; H_4folate, tetrahydrofolate; EDTA, ethylenediaminetetraacetic acid.

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Scheme I[a]

direct transfer

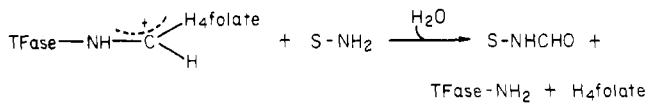
$$S\text{-}NH_2 + 10\text{-}CHO\text{-}H_4folate \xrightarrow{TFase} S\text{-}NHCHO + H_4folate$$

indirect transfer

$$10\text{-}CHO\text{-}H_4folate + X\text{-}TFase \longrightarrow HCOX\text{-}TFase + H_4folate$$

$$HCOX\text{-}TFase + S\text{-}NH_2 \longrightarrow S\text{-}NHCO + TFase\text{-}X$$

or



[a] S = GAR or AICAR.

by the acceptors (GAR, AICAR) on these covalent species would then complete the transfer (see Scheme I). The reaction pathway involving dehydration is chemically distinguishable since the formyl oxygen is lost upon amidine formation and is replaced by solvent oxygen in the products (provided exchange with water is allowed) whereas in the other formyl transfer sequences the formyl oxygen will be retained. The operation of the dehydration mechanism can be determined by utilization of $H_2{}^{18}O$ and formyl ^{16}O where incorporation of ^{18}O into products will indicate amidine formation.

Recently it has been showed that ^{18}O causes an upfield isotope shift on the ^{13}C NMR signal of a carbon bound to that oxygen (Vederas, 1980; Risley & VanEtten, 1979; Diakur et al., 1980). For an amide carbon this shift is approximately 0.03 ppm upfield from the ^{13}C–^{16}O resonance, allowing determination of ^{18}O incorporation by observation of the ^{13}C spectra of the products. If the cofactor for the transformylases were capable of internal amidine formation, e.g., 5,10-methenyl-H_4folate, then ^{18}O incorporation might occur in the transformylase products regardless of the reaction mechanism. However, fully oxidized cofactor analogues such as 10-formyl-5,8-deazafolate for GAR TFase and 10-formyl-8-deazafolate for AICAR TFase serve as excellent replacements for the natural cofactor (Smith et al., 1981b) and, importantly, are incapable of internal amidine formation.

Employing these cofactor analogues, we have used ^{18}O incorporation and ^{13}C-enriched formyl units to investigate the mechanisms of the two transformylases of purine biosynthesis. Furthermore, the possibility that a formyl enzyme intermediate might exist as an anhydride or thioester was investigated by employing hydroxylamine as a trapping agent.

Experimental Procedures

Materials

Potassium [^{13}C]formate (90 atom %), [^{13}C]formaldehyde (90 atom %), and $H_2{}^{18}O$ (99 atom %) were obtained from KOR Isotopes, while potassium [^{13}C]cyanide (90 atom %) was purchased from Merck and [^{14}C]formaldehyde was purchased from New England Nuclear. All solvents were distilled prior to use. Other reagents were used without further purification. GAR TFase, AICAR TFase, GAR, and AICAR were prepared as described previously (Smith et al., 1980; Mueller & Benkovic, 1981). 5,8-Deazafolate and 8-deazafolate were obtained through the generosity of Dr. John Hynes, College of Pharmacy, Medical University of South Carolina, and the Southern Research Institute, Birmingham, AL, respectively. L(−)-H_4folate was prepared enzymatically from H_2folate by dihydrofolate reductase (Mathews & Huennekens, 1960) and purified on DEAE-cellulose.

Methods

[^{13}C]Formylation of 5,8-Deazafolate. Potassium [^{13}C]-formate (16.1 mg, 0.19 mmol, dried in vacuo at 56 °C) was mixed with 50 μL of glacial acetic acid and 11 μL of acetic anhydride. This solution was heated with stirring for 15 min at 55 °C. To this was added 5,8-deazafolate (5.5 μmol) and 150 μL of acetic acid, and the resulting solution was stirred for 20 min. Reaction was judged complete by a change in the UV spectrum from λ_{max} = 295 nm to λ_{max} = 255 nm (Smith et al., 1981b). The product was lyophilized and washed 3 times with 150 μL of ice-cold H_2O. The product gave the UV spectrum of 10-CHO-5,8-deazafolate and was totally converted to the parent free base upon assay by GAR TFase. 10-CH_3CO-5,8-deazafolate, which has a UV spectrum similar to that of 10-CHO-5,8-deazafolate and might be expected to be the major contaminant from the formylation reaction, is not a cofactor for the transformylase (G. K. Smith, unpublished results).

Enzymatic Synthesis of [^{13}C,^{18}O/^{16}O]FGAR. The reaction was run in 1 mL with 30% $H_2{}^{18}O$ in 50 mM Tris-HCl, pH 7.5, containing 5.5 μmol of $NADP^+$, 8 μmol of GAR, 20 μmol of glucose, 12.3 μmol of [^{13}C]formaldehyde, 0.1 μmol of L-(−)-H_4folate, 14 units of glucose oxidase, 11 000 units of catalase, 0.02 unit of GAR TFase, and 0.1 unit of 5,10-methylene-H_4folate dehydrogenase. The dehydrogenase activity is contained in the trifunctional protein, which also catalyzes the hydrolysis of 5,10-CH^+-H_4folate to 10-CHO-H_4folate (cyclohydrolase activity) and the synthesis of 10-CHO-H_4folate from H_4folate (synthetase activity) [see Smith et al. (1980) for details of its purification]. All solutions had been thoroughly degassed under Ar and the glucose oxidase–catalase couple was used to scavenge O_2. After 15 h at 37 °C, 0.6 mL of Me_2SO-d_6 was added, and the solution was filtered into a 10-mm NMR tube and made 2 mM in EDTA. The experiment was preceded by a control employing [^{14}C]-formaldehyde under identical conditions, but in the absence of $H_2{}^{18}O$, to ensure that the above conditions could generate a sufficient amount of FGAR for NMR analysis. The FGAR produced was isolated and quantitated as described previously (Smith et al., 1981b). The results indicated that a 1-mL solution 1.3 mM in FGAR could be made.

[^{13}C,^{18}O/^{16}O]Formylation of 8-Deazafolate. A total of 100 mg (1.5 mmol) of potassium [^{13}C]cyanide was dissolved in 500 μL of 30% $H_2{}^{18}O$ and sealed in a thick-walled tube. Hydrolysis to formate was accomplished by heating at 130 °C for 3 h. Acidification of the hydrolysate with 1 N H_2SO_4 was followed by lyophilization. The volatile material was collected, thawed, and neutralized with 1 N NaOH. Lyophilization of this solution gave 93 mg (91%) of sodium [^{13}C,^{18}O/^{16}O]formate. Conversion to formic–acetic anhydride was accomplished by shaking a suspension of the sodium formate (11.6 mg, 0.17 mmol) in 700 μL of dry ether containing 1 equiv of acetyl chloride for 5 h (Muramatsu et al., 1965). Sodium chloride was removed by centrifugation and the ether was condensed to ~50 μL by careful evaporation. To this partially purified mixed anhydride was added a solution of 5.3 μmol of 8-deazafolate in 500 μL of AcOH and the resulting solution was stirred at 50 °C for 20 min. HPLC analysis (Whatman Partisil PXS 10/25 ODS-2 reverse-phase column, eluant 8% CH_3CN in 0.1 M NH_4OAc, pH 3.6) coupled with the UV spectrum (pH 3.6, 8-deazafolate λ_{max} = 296 nm, 10-CHO-8-deazafolate λ_{max} = 254 nm) indicated essentially quantitative formylation. The sample was prepared for NMR analysis by lyophilization, followed by dissolving the resulting white solid in 1.8 mL of D_2O containing 2 mM EDTA.

[^{13}C]Formylation of AICAR. The method of Mueller & Benkovic (1981) was employed for the synthesis of [*formyl*-^{13}C]FAICAR.

Enzymatic Synthesis of [^{13}C]*FGAR from GAR and* [^{13}C]*-10-CHO-5,8-deazafolate.* The reaction mixture contained, in a total volume of 1.0 mL of 30% $H_2{}^{18}O$, 3.5 μmol of [^{13}C]-10-CHO-5,8-deazafolate, 15.2 μmol of α,β-GAR, 50 μmol of Tris-HCl, pH 7.5, 1 μmol of EDTA, and 70 μg of GAR TFase (0.024 unit). The reaction was allowed to proceed at 25 °C for 22 h after which it was judged complete by its UV spectrum (Smith et al., 1981b). To the product solution was added 0.6 mL of Me_2SO-d_6 for a field-frequency lock, and the sample was analyzed by ^{13}C NMR.

Enzymatic Synthesis of [^{13}C]*-10-CHO-8-deazafolate from* [^{13}C]*FAICAR.* The reaction was performed at 25 °C in a total volume of 1.7 mL containing 119 μmol of Tris-HCl, pH 7.5 (in 30% $H_2{}^{18}O$), 39 μmol of [^{13}C]FAICAR, 3.7 μmol of 8-deazafolate, and 51 μmol of KCl. Reaction was initiated by the addition of 0.06 unit of AICAR TFase and monitored at 325 nm in 1-mm path length cells. The reaction was quenched with 1 N KOH to a final pH of 9.1, and Me_2SO-d_6 was added to a final concentration of 30% by volume. 10-CHO-8-deazafolate was quantitated by HPLC (see above under the synthesis of the control for conditions) and by its UV spectrum and was found to be at a concentration of 1 mM.

Nuclear Magnetic Resonance Spectroscopy. ^{13}C NMR spectra were obtained at 90.56 MHz by using a Brüker Instruments Inc. WM-360. Field-frequency locking to ^2H was maintained by using either Me_2SO-d_6 or D_2O. For optimization of digital resolution, the observation frequency was centered close to the resonance of interest, and the sweep width was set at 2000 Hz (32K data points, acquisition time 8.19 s) at ambient probe temperature under conditions of broadband proton decoupling. Gaussian multiplication of the free induction decay was employed to enhance resolution. Chemical shifts are reported in parts per million relative to Me_4Si, with Me_2SO-d_6 as a secondary internal standard (39.60 ppm).

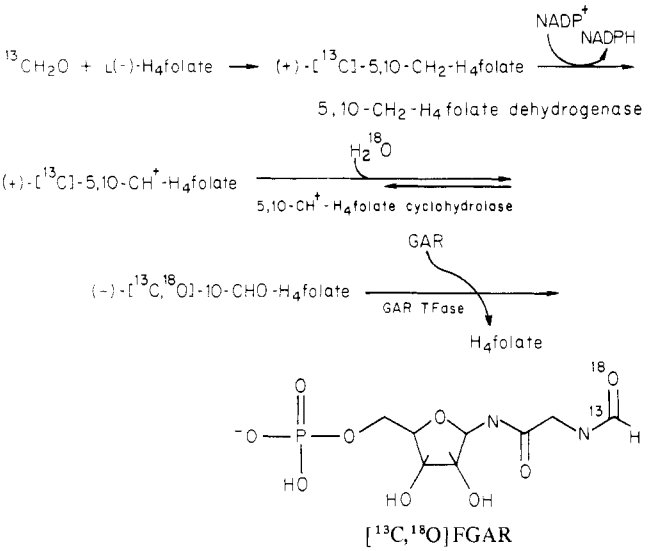
Stoichiometry of AICAR and GAR TFase in the Presence of Hydroxylamine. The stoichiometry of the AICAR TFase reaction was determined in the presence of 1 mM hydroxylamine hydrochloride, pH 7.5, by the procedure of Mueller & Benkovic (1981).

The stoichiometry of the GAR TFase reaction was determined by modification of the continuous GAR TFase assay (Smith et al., 1981b). The cuvette contained 13.6 μmol of sodium maleate, pH 6.8, 0.086 μmol of EDTA, 0.4 μmol of hydroxylamine hydrochloride, 0.2 μmol of α,β-GAR, and 0.020 μmol of [*formyl*-^{14}C]-10-CHO-H_4folate (sp act. 2.0 μCi/μmol) in 0.90 mL under N_2 in a microcuvette. The base-line rate at 312 nm was recorded at 25 °C in a thermostated Gilford spectrophotometer for 10 min, and the reaction was started by addition of 3.6×10^{-4} unit of GAR TFase. The reaction was monitored at 312 nm and was quenched by addition of 70 μL of 0.5 M NaOH after which [^{14}C]FGAR was purified by passage through a QAE-Sephadex column (0.5 × 12.5 cm, equilibrated with 0.01 M Na_2CO_3), eluting with 0.02 M Na_2CO_3, pH 9.9 (Smith et al., 1981b).

Results and Discussion

The possible incorporation of solvent oxygen into FGAR or FAICAR, which would implicate the formation of an amidine intermediate, was investigated by attempting to observe the ^{18}O-induced isotope shift of the ^{13}C resonance position of the formyl carbon. To determine if this shift is observable, it was necessary to first synthesize the desired formylated species enriched in both ^{13}C and ^{18}O. For the study of GAR TFase, it was necessary to synthesize [*formyl*-^{13}C]FGAR with a known ^{18}O enrichment in the formyl oxygen. This was best accomplished enzymatically as shown in Scheme II. A test

Scheme II



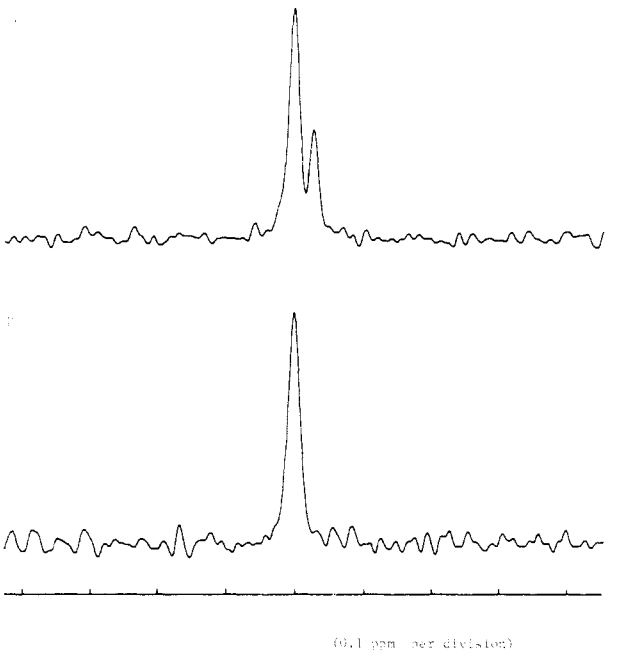
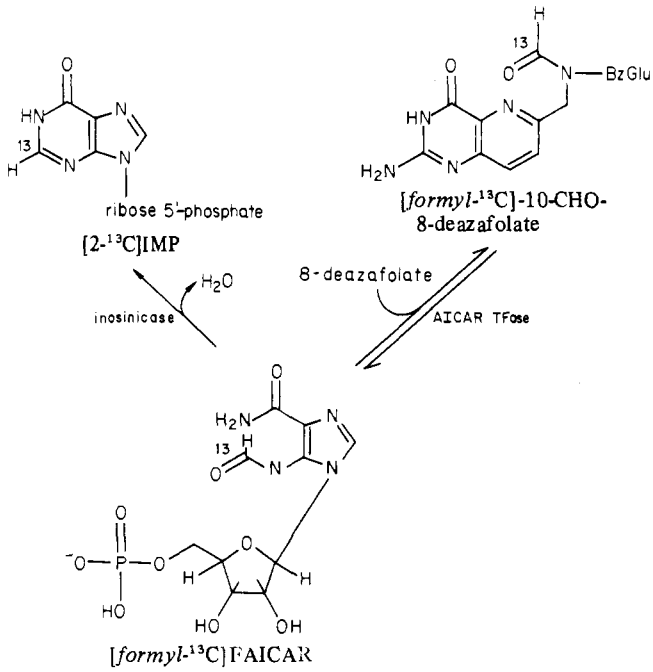


FIGURE 1: ^{13}C NMR spectra of (A) [*formyl*-^{13}C,^{18}O/^{16}O]FGAR (30% ^{18}O enriched, 4345 transients) and (B) [*formyl*-^{13}C]FGAR enzymatically synthesized from [*formyl*-^{13}C]-10-CHO-5,8-deazafolate in 30% $H_2{}^{18}O$ (344 transients); see Experimental Procedures for further details. Limit of detection of ^{18}O incorporation is estimated at 5% (based on a calculated S/N ratio).

reaction was first carried out with [^{14}C]formaldehyde and the [^{14}C]FGAR isolated. It was found that 1.3 mM FGAR (in 1.0 mL) could be produced. The experiment was then repeated with [^{13}C]formaldehyde in 30% $H_2{}^{18}O$. Upon completion of the reaction, Me_2SO-d_6 was added to a final concentration of 38%, and the NMR analysis was performed. The result is shown in Figure 1A. It can be seen that two resonances are observed with half-height resolution. The chemical shifts (relative to Me_4Si) of the [^{16}O]- and [^{18}O]formyl species are 165.415 and 165.387 ppm, respectively. Thus, under these conditions, the ^{18}O-induced isotope shift of 0.028 ppm can be readily observed.

To determine if an enzyme–folate (or GAR–folate) amidine forms during catalysis, it is necessary to use a 10-formyl cofactor which cannot form an internal amidine, i.e. 5,10-methenyl-H_4folate, since this would lead to solvent oxygen exchange regardless of the mechanism of transformylation.

Scheme III[a]



[a] BzGlu = p-$C_6H_4CONHCH(CO_2H)(CH_2)_2CO_2H$

5,8-Deazafolate was formylated at N-10 with [^{13}C]formic–acetic anhydride. In the presence of excess GAR in 30% $H_2{}^{18}O$ (pH 7.5)–50 mM Tris-HCl, GAR TFase totally converted the cofactor to the free base and produced [^{13}C]FGAR of unknown ^{18}O enrichment. The ^{13}C NMR spectrum of this sample is shown in Figure 1B. Only one carbon resonance is visible in the formyl region, and at this signal to noise level and line width, a second peak, 41% of the first in area and 0.028 ppm upfield, would be seen. Therefore, no solvent oxygen incorporation occurred.

The corresponding experiment for AICAR transformylase was also performed with two major modifications. First, the product of the reaction in the physiologically important direction, namely, FAICAR, is rapidly cyclized by inosinicase to IMP. This activity is apparently on the same peptide as the transformylase and cannot be separated (unpublished results). Therefore, in order to prevent irreversible loss of formyl oxygen, it is necessary to start with FAICAR and formylate a folate analogue in the reverse direction in direct competition with the inosinicase activity (see Scheme III). Second, the oxidized folate analogue which has the greater V_{max}/K_m for AICAR transformylase is 8-deazafolate (Smith et al., 1981a). Although this compound does possess a 5 nitrogen it cannot form an internal amidine since the pyrazine ring is fully oxidized.

In order to show that the ^{18}O-induced isotope shift can be observed, [*formyl*-^{13}C]-10-CHO-8-deazafolate containing a known enrichment of ^{18}O in the formyl position was synthesized. Hydrolysis of potassium [^{13}C]cyanide in 30% $H_2{}^{18}O$ gave formate enriched to 25% in ^{18}O (by NMR integration of the $^{16}O_2$-, ^{16}O-, ^{18}O-, and $^{18}O_2$-shifted ^{13}C resonances). This material was, in turn, converted to the mixed formic–acetic anhydride and used to formylate 8-deazafolate. NMR analysis of this compound showed two resonances in the formyl region at 165.90 ppm (^{16}O) and 165.87 ppm (O^{18}) with an ^{18}O incorporation of 22%. The isotope shift was observed to be 2.7 Hz (0.030 ppm) at 90.56 MHz (see Figure 2A).

To determine if the enzyme-catalyzed transformylation incorporates solvent oxygen, we converted [*formyl*-^{13}C]FAICAR to a mixture of IMP and 10-CHO-8-deazafolate in 30% $H_2{}^{18}O$. The result, as shown in Figure 2B, is that no solvent oxygen incorporation occurs.

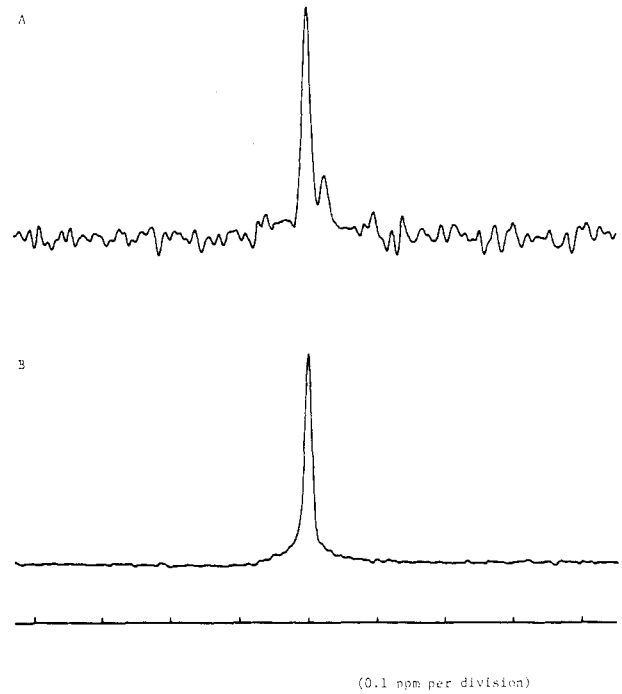


FIGURE 2: ^{13}C NMR spectra of (A) [*formyl*-^{13}C,^{18}O/^{16}O]-10-CHO-8-deazafolate (22% ^{18}O enriched, 1845 transients) and (B) [*formyl*-^{13}C]-10-CHO-8-deazafolate enzymatically synthesized from 8-deazafolate and [*formyl*-^{13}C]FAICAR in 30% $H_2{}^{18}O$ (300 transients); see Experimental Procedures for further details. Limit of detection of ^{18}O incorporation is estimated at 2% (based on a calculated S/N ratio).

If an enzyme-bound formate in the form of a thioester or an anhydride were an intermediate in either of the transformylase reactions, then 1 mM hydroxylamine might react with that species and render it incapable of further formylation (Inward & Jencks, 1965; Epand & Wilson, 1965). Uncoupling by hydroxylamine would disrupt the 1:1 stoichiometry that is found for both transformylases (Mueller & Benkovic, 1981; Caperelli et al., 1980). For AICAR TFase at the lower concentration of AICAR, 25.2 μM (–)-H_4folate and 32.7 μM IMP were produced, while at the higher AICAR concentration 40.2 μM H_4folate and 45.8 μM IMP were produced. Thus the ratio of IMP to H_4folate is 1.2:1, indicating that the stoichiometry of the AICAR TFase reaction is unchanged. For GAR TFase 15.7 μM H_4folate and 16.2 μM FGAR were produced, again indicating no change in the 1:1 stoichiometry. Although these data suggest the absence of a formyl enzyme intermediate, they are by no means conclusive. It is possible that an alternate nucleophile such as hydroxylamine has no access to the active site or that the enzyme-bound formyl intermediate is too short-lived to be kinetically trapped.

Transfer of the formyl group from cofactor to enzyme and then to substrate also is not supported by kinetic data that indicate a sequential reaction mechanism for both enzymes (Caperelli et al., 1980; Mueller & Benkovic, 1981) instead of the expected ping-pong kinetics (Lehninger, 1975).

In conclusion, the most reasonable mechanism for the transformylase-catalyzed reactions based on the available information involves the direct transfer of the formyl group between the amino functions of H_4folate and GAR or AICAR. The design of potential tightly bound reversible inhibitors will be predicated on the basis of this mechanism.

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Small-Angle Neutron Scattering Study of Bence–Jones Protein Mcg: Comparison of Structures in Solution and in Crystal†

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ABSTRACT: Immunoglobulins and immunoglobulin fragments are composed of globular domains linked by extended polypeptide segments. The molecular flexibility inherent in this arrangement allows for significant potential differences between structures observed in the crystalline state and those attained in solution. Small-angle neutron scattering measurements in dilute solution were performed on the Mcg Bence–Jones protein dimer, for which accurate atomic coordinates have been determined by crystallographic methods [Edmundson, A. B., Ely, K. R., Abola, E. E., Schiffer, M., & Panagiotopoulos, N. (1975) *Biochemistry 14*, 3953–3961; Schiffer, M. (1980) *Biophys. J. 32*, 230–232]. The measured radius of gyration (R_g) in H_2O buffer is 24.0 ± 0.4 Å and in D_2O buffer is 23.3 ± 0.1 Å; the calculated value of R_v (R_g in vacuo) is 24.0 Å. The above values compare well with the calculated R_g value of 23.6 Å when refined coordinates of the trigonal crystal form of the Mcg Bence–Jones protein are used. On the basis of a match point of 44.2% D_2O concentration, the experimental partial specific volume is 0.74 cm^3/g. The experimentally derived molecular weight of 47 000 is in very good agreement with that (45 500) calculated from the amino acid composition. For comparisons with different Fab's (antigen binding fragments) exhibiting various "elbow bends" due to the flexibility of the switch peptide between variable and constant domains of the immunoglobulin chains, calculation of the R_g value of the Mcg dimer was performed as a function of the elbow bend. The R_g varied from 22.8 to 26.0 Å as the elbow bend was opened from 100° to 180°; the maximum radius of gyration of the particle was 26.5 Å with the switch peptide stretched by separating the variable and constant domains by an additional 1.5 Å at an elbow bend of 180°.

The correspondence of the functional structure of a molecule in solution and the structure determined by X-ray crystallography has been the subject of continued discussion. This question is especially pertinent for immunoglobulins (Ig's), which are composed of several globular domains linked by extended polypeptide segments. The domain structure imparts an inherent flexibility to the Ig molecule and its Fab (antigen binding) and Fc components. This flexibility may be necessary for the multifunctional nature of the protein, and thus, in contrast to most globular enzymes, the Ig's might be expected to differ between the crystalline and solution states in their gross conformation. Complete IgG immunoglobulins and their fragments have been studied by single-crystal X-ray diffraction (Poljak et al., 1973; Segal et al., 1974; Matsushima et al., 1978; Navia et al., 1979; Silverton et al., 1977; Marquart et al., 1980) and by small-angle X-ray (Pilz et al., 1973, 1975, 1976, 1977) and neutron scattering (Cser et al., 1976; Gilmour et al., 1981) in dilute solution. The overall dimensions of the molecules derived by these two methods differ significantly, the values obtained in solution being larger.

The IgG molecules consist of two heavy and light chains; the light chains and the N-terminal half of the heavy chains form the antigen binding (Fab) fragments. Each chain with the Fab's consists of an N-terminal variable and a C-terminal constant domain connected by the "switch" peptide. Light chains also form dimers and are known as Bence–Jones proteins. The Mcg Bence–Jones protein consists of two chemically identical human λ-type light chains and resembles an Fab both in its three-dimensional structure and in its ability to bind small molecule haptens (Schiffer et al., 1973; Edmundson et al., 1974). The structure of the Mcg dimer in the trigonal-crystal form was determined at atomic resolution (Edmundson et al.,

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