Characterization of the Enzyme Complex Involving the Folate-Requiring Enzymes of de Novo Purine Biosynthesis†

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ABSTRACT: Evidence is presented for a functional association of GAR TFase and the trifunctional protein within the protein complex consisting of GAR TFase, AICAR TFase, Ser HMase, and trifunctional protein. Resolution of the trifunctional protein from the remaining enzymes in the complex causes a loss of GAR TFase activity which is regained upon recombination. The minimum stoichiometry for GAR TFase reactivation is 3:1 GAR TFase–trifunctional protein. Determination by ultracentrifugation of the sedimentation coefficient as a function of protein concentration disclosed that the complex is in mobile equilibrium with the individual proteins. However, mixed GAR TFase–trifunctional protein species can be detected by trapping with cleavable bifunctional cross-linking reagents. Additional support for their interaction is found in the kinetic coupling of the trifunctional protein and GAR TFase activities that leads to a fourfold more efficient formation of formylglycinamide ribotide commencing with formate rather than with 5,10-methenyl-H_4folate triglutamate.

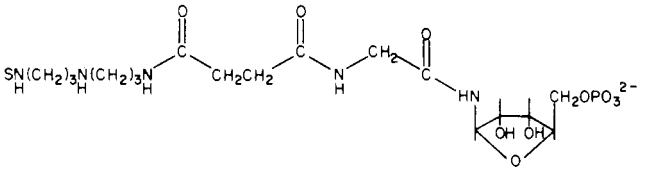
In a recent publication we reported the purification of glycinamide ribotide transformylase[1] via a mild procedure which led to the copurification of three other enzymes: the trifunctional 5,10-methenyl-, 5,10-methylene-, and 10-formyl-H_4folate synthetase, aminoimidazolecarboxamide ribotide transformylase, and serine transhydroxymethylase, all of which use reduced folate cofactors (Caperelli et al., 1980). The results suggested a specific association between the enzymes such that they might function in vivo as an enzyme complex. In the present paper we present direct proof for the interaction between two of the enzymes.

Experimental Procedures

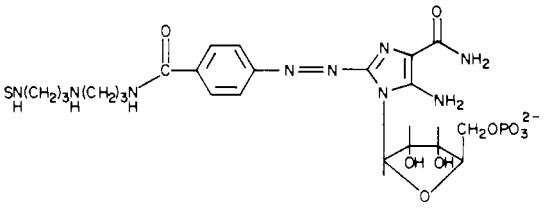
Materials

α,β-Glycinamide ribotide (GAR) was prepared by the method of Chettur & Benkovic (1977). 5-Aminoimidazole-4-carboxamide-β-D-ribofuranosyl 5′-monophosphate (Ba^{2+} salt) (AICAR) was prepared by phosphorylation of the corresponding riboside (Yoshikawa et al., 1967; Murray & Atkinson, 1968). AICAR riboside, tetrahydrofolic acid (H_4-folate), pig heart fumarase, yeast alcohol dehydrogenase, rabbit muscle pyruvate kinase, yeast glucose-6-phosphate dehydrogenase, and bovine pancreatic ribonuclease were purchased from Sigma Chemical Co., St. Louis, MO. Pteroyl-(γ-L-glutamyl)$_2$-L-glutamic acid was prepared by the method of Baugh et al. (1970) and converted enzymatically to the (−)-L-H_4folate(Glu)$_3$ according to the methods of Blakley (1960) and Mathews & Huennekens (1960). (±)-L-5,10-C^+H-H_4folate and (+)-L-5,10-C^+H-H_4folate(Glu)$_3$ were prepared as described by Rowe (1968). Sodium [^{14}C]formate (59 mCi/mmol) was purchased from Amersham/Searle. GAR Sepharose was synthesized as previously described (Chettur, 1977). The structure is shown in Scheme I. NADP Sepharose was prepared by the technique of Lamed et al. (1973). Sephadex G-200 (fine) and Sepharose 4B were purchased from Pharmacia Fine Chemical, Uppsala, Sweden. Bio-Gel P-300 (50–100 mesh) and all electrophoresis chemicals were products of Bio-Rad Laboratories, Richmond, CA. All other chemicals were of the highest grade commercially available and purified by standard techniques prior to use.

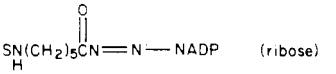
Scheme I: Affinity Column Ligands



GAR-Sepharose



AICAR-Sepharose



NADP-Sepharose

AICAR–Sepharose was synthesized via the following diazo coupling technique (Cohen, 1974) (Scheme I). Sepharose 4B (40 g) was activated at 0 °C with 8 g of CNBr at pH 11 (maintained with 1 M NaOH) according to the procedure of Nishikawa & Bailon (1975). The washed gel cake was suspended in 20 mL of 0.2 M $NaHCO_3$ containing 6 mL of 3,3′-diaminodipropylamine, pH 10.0, and shaken for 16 h at room temperature. The coupled gel was then washed with 500 mL of H_2O, 0.2 M acetic acid, H_2O, 0.5 M NaOH, H_2O, 0.2 M acetic acid, and 2 L of H_2O in that order. A portion of the gel (20 g) was shaken with 20 mL of 1 M ethanolamine

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[1] Abbreviations used: GAR, glycinamide ribotide; FGAR, *N*-formylglycinamide ribotide; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate; H_4folate, tetrahydrofolate; H_4folate(Glu)$_3$, tetrahydrofolate triglutamate; 2-ME, 2-mercaptoethanol; Me_2SO, dimethyl sulfoxide; GAR TFase, GAR transformylase; AICAR TFase, AICAR transformylase; Ser HMase, serine transhydroxymethylase; trifunctional protein, 5,10-methenyl-, 5,10-methylene-, and 10-formyl-H_4folate synthetase (combined); NADP, nicotinamide adenine dinucleotide phosphate; HTP, hydroxylapatite cellulose; $NaDodSO_4$, sodium dodecyl sulfate; DTBP, dimethyl 3,3′-dithiobis(propionimidate); BSA, bovine serum albumin.

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at pH 8.3 for 3 h at room temperature. It was then collected by filtration, washed with 2 L of H_2O, and dried by suction.

The activated gel was suspended in a solution consisting of 24 mL of 0.2 M sodium borate, pH 9.4, and 16 mL of dimethylformamide (DMF) and cooled at 4 °C. To this was added 0.576 g of *p*-nitrobenzoyl azide in 7 mL of DMF dropwise with stirring at 4 °C. The suspension was stirred for 1.5 h at 4 °C and 7 h at room temperature. The resulting yellowish gel was washed sequentially with 1.5 L of 1:1 DMF–H_2O and 2 L of H_2O. For reduction of the nitrophenyl group to the corresponding amine, a suspension of the gel was stirred in 30 mL of a solution containing 0.2 M sodium dithionite and 0.5 M $NaHCO_3$, pH 8.5, for 2 h at 40–45 °C, filtered, and washed with 2 L of H_2O.

For diazotization ice-cold 0.5 M HCl (30 mL) and 0.1 M $NaNO_2$ (30 mL) were added to the moist gel cake, and the suspension was stirred at 0 °C for 7 min. The gel was collected, dried by suction, washed with 1.5 L of cold H_2O, and then resuspended in 30 mL 0.2 M sodium acetate, pH 5.9, at 4 °C. To the suspension was added 0.5 mmol of AICAR (Ba salt), and the suspension was stirred for 1 h at room temperature. The gel was collected by filtration and washed sequentially with 1 L of H_2O, 1 L of a solution of 0.5 M KCl and 50 mM Tris-HCl (pH 8.0), and 2 L of H_2O. The AICAR incorporation was determined by assaying for organic phosphate according to the method of Chen et al. (1956). A typical analysis showed 12 μmol of phosphate/g of gel.

The crude enzyme complex was prepared by the technique of Caperelli et al. (1980). Fractions eluted from the second HTP column with an ATP (5–15 mM) gradient were further purified by affinity chromatography.

Methods

Enzyme Assays. Assays of GAR TFase, 5,10-C^+H-H_4folate cyclohydrolase, 10-CHO-H_4folate synthetase, and 5,10-CH_2-H_4folate dehydrogenase were performed according to Caperelli et al. (1980) with the following exceptions: (1) in the synthetase assay, 2 mM ATP, 4 mM $MgCl_2$, 10 mM sodium formate, and 0.4 mM H_4folate were used; (2) in the dehydrogenase assay a 20% excess of formaldehyde over H_4folate was employed. Ser HMase was assayed with β-phenylserine by recording the increase in A_{279} on the production of benzaldehyde (Schirch & Diller, 1971). AICAR TFase was assayed according to the method of Black et al. (1978) Protein concentration was determined by using a UV biuret technique (Zamenhof, 1957). A standard curve was established with four proteins: RNase A, bovine serum albumin, ovalbumin, and γ-globulin. All four gave identical standard curves.

Affinity Chromatography. The partially purified enzyme loaded onto the AICAR–Sepharose affinity column was from an ATP elution of the second HTP column, which had been desalted on Sephadex G-25 and dialyzed against the equilibrating buffer (Caperelli et al., 1980). All chromatography buffers contained 10 mM 2-ME, 25% glycerol, and 10% Me_2SO, subsequently referred to as "stabilizers".

Columns of AICAR–Sepharose (1–20 mL) were prewashed with several column volumes of 75 mM Tris-HCl and 75 mM K_2HPO_4, pH 7.5, plus stabilizers and then equilibrated at 4 °C with 7.5 mM Tris-HCl, pH 7.5, plus stabilizers. The enzyme was loaded onto the column in the same buffer, and the column was then washed with 37.5 mM Tris-HCl, pH 7.5, plus stabilizers until the A_{280} was constant and low. The purified complex was then eluted with a solution of 75 mM Tris-HCl and 75 mM K_2HPO_4, pH 7.5, containing stabilizers. Active fractions were pooled and Me_2SO was removed by dialysis against 7.5 mM K_2HPO_4, pH 7.5, plus 25% glycerol and 10 mM 2-ME. Finally the enzyme was concentrated to 0.5–2 mg/mL (A_{280}) if necessary on an Amicon YM-10 ultrafiltration membrane and stored in liquid nitrogen.

NADP–Sepharose was precycled as with the AICAR–Sepharose, equilibrated with 7.5 mM Tris-HCl, pH 7.5, plus stabilizers, and loaded with enzyme fractions obtained from elution of the AICAR–Sepharose or the HTP column. The column was then washed with more of the equilibrating buffer until the A_{280} was constant and low. GAR TFase, AICAR TFase, and Ser HMase were then coeluted with 100 mM Tris-HCl, pH 7.5, plus stabilizers. Finally, pure trifunctional protein was eluted with 75 mM Tris-HCl, and 75 mM K_2HPO_4, pH 7.5, plus stabilizers. Fractions containing the three activities and fractions containing the trifunctional protein were dialyzed against 7.5 mM K_2HPO_4, pH 7.5, containing 25% glycerol and 10 mM 2-ME and concentrated to 1–2 mg/mL in the former and 0.5 mg/mL in the latter case.

Gel Filtration. A 110 × 1.8 cm Sephadex G-200 column was equilibrated at 4 °C with 50 mM K_2HPO_4, pH 7.5, containing stabilizers. The column pressure head was maintained by 15–18 cm of buffer with a flow rate of 3 mL/h. A 3-mL solution of the complex (0.22 GAR TFase unit) purified by AICAR–Sepharose chromatography also containing 0.5 mg each of rabbit muscle pyruvate kinase, pig heart fumarase, yeast glucose-6-phosphate dehydrogenase, yeast alcohol dehydrogenase, and bovine pancreatic RNase A as molecular weight standards was layered on the column and eluted with the equilibrating buffer. One-milliliter fractions were collected.

A 115 × 1.8 cm Bio-Gel P-300 column was equilibrated at 4 °C with 50 mM K_2HPO_4, pH 7.5, plus stabilizers. The column pressure head was maintained by 15–20 cm of buffer with a flow rate of 7.2 mL/h. The other experimental protocol was identical with that described for Sephadex G-200.

Sucrose Density Gradient Ultracentrifugation. This technique was performed by the method of Martin & Ames (1961) in 5–20% sucrose gradients (33 mL of total volume). Gradients were poured at room temperature and allowed to equilibrate at 4 °C overnight. Centrifugation was performed on 0.2–0.75 mL of enzyme at 4 °C in a Beckman Model L5-50 preparative ultracentrifuge equipped with a SW-27 swinging bucket rotor. Centrifugation at 25 000 rpm was carried out for 48 h. Molecular weights were determined by using yeast alcohol dehydrogenase as an internal standard.

Sedimentation Velocity. These experiments were accomplished on a Beckman-Spinco Model E analytical ultracentrifuge equipped with UV optics and scanner. Centrifugation at 55 000 rpm was performed at 20 °C in 75 mM K_2HPO_4, 10 mM 2-ME, and 25% glycerol, pH 7.5. The viscosity and density of the solution at 20 °C were 2.51 cP and 1.073 g/mL, respectively.

Protein concentrations of 0.1–1.5 mg/mL were used in the presence of the substrate GAR (0.25 mM), and concentrations of 0.5 to 2.0 mg/mL were used in the absence of GAR. Enzyme used in these experiments had been purified through AICAR–Sepharose. Sedimentation coefficients were calculated from plots of ln r vs. time (Tanford, 1961).

Cross-Linking with DTBP. Enzyme at a concentration of 0.5 mg/mL was dialyzed against deaerated, N_2-saturated 50 mM K_2HPO_4 and 25% glycerol, pH 7.5, under an N_2 atmosphere. The protein mixture was composed of 3:1 GAR TFase to trifunctional protein obtained by combining appropriate fractions obtained from NADP–Sepharose chromatography. The pH was then adjusted to 8.3 with 1 N KOH, and 0.25 mg of DTBP was added per mL of enzyme solution. The pH

Table I: Activity Ratios[a] before and after AICAR-Sepharose

enzyme	activity ratio					
	10-formyl-H_4folate synthetase	5,10-methylene-H_4folate dehydrogenase	5,10-methenyl-H_4folate cyclohydrolase	AICAR transformylase	Ser HMase	GAR transformylase
ratio before	10.9	6.51	6.5	0.46	8.6	1
ratio after	15.6	11.8	6.3	0.34	12.2	1

[a] All activities are relative to GAR transformylase.

was then readjusted to 8.3. After 45 min under an atmosphere of N_2, 1.0 mg of solid $NaDodSO_4$ was added per mL of reaction mixture, and the enzyme was denatured 2 h in the absence of 2-ME at 37 °C under an atmosphere of N_2. Cross-links were cleaved after the first dimension of electrophoresis with 10% 2-ME in $NaDodSO_4$ gel buffer.

$NaDodSO_4$ Gel Electrophoresis. One-dimensional electrophoresis employed 5 or 7.5% polyacrylamide gels that were run at 8 mA/gel according to Weber et al. (1972). Two-dimensional $NaDodSO_4$ gel electrophoresis on 5 or 7.5% gel was carried out as described by Coggins et al. (1976). The slab gel apparatus was constructed in our laboratory according to a modification of the apparatus of Driedger & Blumberg (1978). Coomassie blue staining was performed by the method of Weber et al. (1972). Silver staining of slab gels was performed by the method of Merril et al. (1979).

Recombination Experiments. Solutions of the trifunctional protein ranging in concentration from 0.02 to 1.0 μM were prepared in media buffered with 7.5 mM K_2HPO_4, 25% glycerol, 10 mM 2-ME, and 0.2 mg/mL BSA (pH 7.5). These diluted solutions did not lose enzymic activity overnight. The GAR TFase fraction obtained from NADP–Sepharose elution (92% GAR TFase and 7% trifunctional protein based on densitometer scans of $NaDodSO_4$ gels) was diluted to 0.65 and 0.029 μM in GAR TFase and trifunctional protein, respectively, with the above buffer media immediately before use.

Recombination was commenced by adding 6.75 μL from solutions containing the trifunctional protein to the GAR TFase assay buffer. Then 6, 9, 12, or 15 μL of 0.65 μM GAR TFase was added, and the resulting solution incubated at 37 °C for 10 min before introducing 5,10-methenyl-H_4folate. The final assay volume was 0.675 mL. The GAR TFase reactivation was determined by assaying for its activity. Calculations for the complex dissociation constant (K_d) were based on formal total protein concentrations in the assay medium.

A sample of the purified trifunctional protein (50 μL, 0.528 mg of protein/mL) was diluted to 100 μL with 7.5 mM potassium phosphate, 25% glycerol, and 10 mM 2-ME, pH 8.0. The solution was brought to pH 8.3 by addition of 0.1 M KOH and was made 50 mM in iodoacetamide by the addition of the solid. After the solution was mixed, the reaction was allowed to proceed for 45 min at room temperature. The entire solution was dialyzed against 100 mL of 7.5 mM potassium phosphate, 25% glycerol, and 10 mM 2-ME, pH 8.0 followed by dialysis against 100 mL of 7.5 mM potassium phosphate and 25% glycerol, pH 7.5. The recovered protein solution (50 μL) was used in recombination experiments with the GAR TFase fraction following the above procedure.

Dilution Experiments. The complex eluted from AICAR–Sepharose (2.9 μM GAR TFase and 2.0 μM trifunctional protein based on densitometer scans of $NaDodSO_4$ gels) was dissolved in the GAR TFase assay medium to give concentrations of GAR TFase and trifunctional protein ranging from 12.2 to 0.65 nM and 8.4 to 0.45 nM, respectively, and preincubated for 30 min–3 h before 5,10-methenyl-H_4folate was added to initiate the assay.

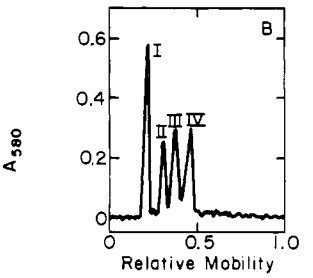


FIGURE 1: $NaDodSO_4$ gel electrophoresis of Tris–phosphate elution from AICAR–Sepharose. Electrophoresis was performed according to the method of Weber et al. (1972) on 7.5% polyacrylamide gels. Protein bands are assigned as follows: I, trifunctional protein; II, AICAR TFase; III, GAR TFase, IV, Ser HMase.

Coupled Kinetic Experiments. Experiments designed to demonstrate coupling of the complex activities were performed under N_2 using a reaction mixture containing 0.1 M NH_4Cl, 2 mM sodium formate, 0.5 mM (α+β)GAR, 4 mM $MgCl_2$, 14 μM H_4folate$(Glu)_3$, and 2 mM ATP in 50 mM maleate, pH 6.8, at 25 °C. In each case the reaction was initiated with 8 μL of enzyme complex: by activities (8.94×10^{-2} unit/mL GAR TFase:1.41 units/mL synthetase) and by densitometer scans of $NaDodSO_4$ gels (1.51 μM GAR TFase and 0.910 μM trifunctional protein). The production of 5,10-methenyl-H_4-folate$(Glu)_3$ was followed by ΔA_{355}. The total of 5,10-methenyl-H_4folate$(Glu)_3$ plus 10-formyl-H_4folate$(Glu)_3$ produced was determined by quenching 50-μL aliquots, removed at various times, in 150 μL of 6 N HCl and reading the A_{350}. The amount of FGAR produced was determined radiochemically by using 2 mM sodium [^{14}C]formate. In this assay 150-μL portions of the reaction mixture were removed at 0, 15, 30, and 60 min and quenched in 150 μL of 0.093 N NaOH. Each quenched sample was applied to a 0.55 × 9 cm column of QAE-Sephadex which had been equilibrated previously with 0.01 M $NaHCO_3$, pH 9.9. [^{14}C]FGAR was eluted with 0.02 M $NaHCO_3$, pH 9.9. Radioactivity measurements were done with a Beckman LS-8100 liquid scintillation counter using Aquasol-2 (New England Nuclear).

Results

Coelution from an AICAR Affinity Column. As has been previously shown, GAR TFase, AICAR TFase, 5,10-methenyl-H_4folate cyclohydrolase, 10-formyl-H_4folate synthetase, 5,10-methylene-H_4folate dehydrogenase, and Ser HMase activities copurify on GAR–Sepharose (Caperelli et al., 1979, 1980). The specific association of the various activities suggested by this result is supported by a similar copurification of the enzymes on a column specific for AICAR TFase. However, since elution has been effected by a nonspecific ligand, phosphate, this finding does not constitute strong supporting evidence for the complex. The activities of the proteins obtained by this latter procedure are listed in Table I; a typical protein gel pattern resulting from elution from AICAR–Sepharose is shown in Figure 1. Both the AICAR– and GAR–Sepharose columns result in purification with

Table II: Specific Activities of the Trifunctional Protein

enzyme	sp act. (units/mg)	x-fold purifn from crude
synthetase	10.3	741
dehydrogenase	12.1	681
cyclohydrolase	30.6	936

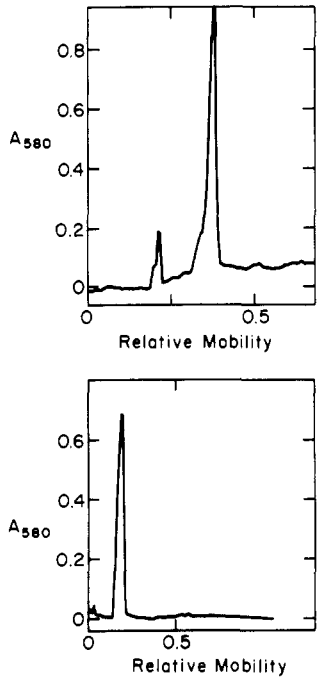


FIGURE 2: Upper: $NaDodSO_4$ gel electrophoresis of Tris elution of NADP–Sepharose, 92% GAR TFase (relative mobility = 0.35), and 7% trifunctional protein (relative mobility = 0.22). Lower: $NaDodSO_4$ gel electrophoresis of Tris–phosphate elution of NADP–Sepharose, 100% trifunctional protein. Electrophoresis was performed according to the method of Weber et al. (1972) on 7.5% polyacrylamide gels.

comparable activity ratios for the same four proteins which have been assigned as I, trifunctional protein, II, AICAR TFase, III, GAR TFase, and IV, Ser HMase (Caperelli et al., 1980). Since the AICAR–Sepharose capacity is typically greater than that of the GAR–Sepharose (the former binds ~2 mg of protein/mL of resin while the latter binds ~0.03 mg/mL), it becomes the preferred method for obtaining these activities.

Chromatography on NADP–Sepharose. Affinity chromatography on NADP–Sepharose of the complex obtained after phosphate (75 mM) elution of the proteins from the AICAR affinity column results in purification to homogeneity of the trifunctional protein and recovery in a subsequent fraction of a protein solution ~92% GAR TFase and 7% trifunctional protein with minor amounts of AICAR TFase and Ser HMase. This composition was estimated from densitometer scans of $NaDodSO_4$ analytical gels assuming equivalent staining of the various proteins. However, the purification step leads to ~80% loss of the GAR TFase activity. The results are summarized in Table II and shown in Figure 2.

Recombination Experiments. Since removal of the trifunctional protein from GAR TFase on NADP–Sepharose leads to inactivation of the GAR TFase, it was reasoned that readdition of the former may lead to reactivation. The result of titrations of four differing concentrations of GAR TFase with the trifunctional protein are presented in Figure 3, showing a large activation (up to 10-fold) of GAR TFase in all four instances upon saturation by the trifunctional protein. A cursory study of the time dependence for reactivation showed

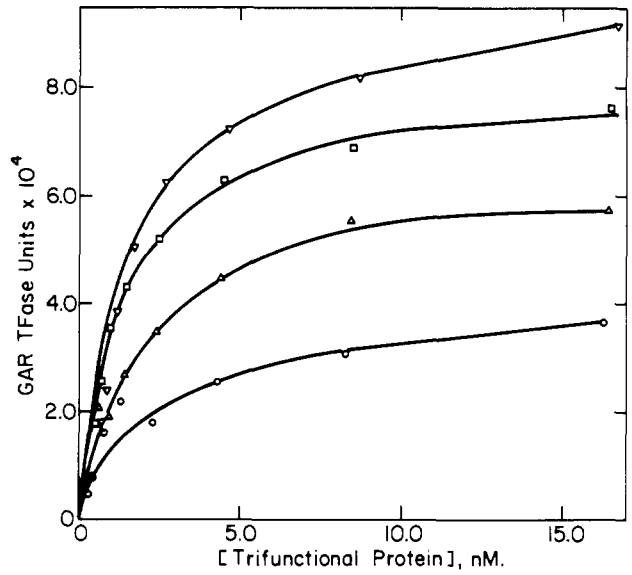


FIGURE 3: Activation of GAR TFase by the trifunctional protein. GAR TFase at concentrations of 5.7 (○), 8.5 (△), 11.4 (□), and 14.2 nM (▽) were recombined in the GAR TFase assay with varying trifunctional protein concentrations. The enzymes were preincubated together at 37 °C for 10 min before 5,10-methenyl-H_4folate was added to start the assay.

the latter to be complete within the dead time of the assay, 10 min. Bovine serum albumin, lactate dehydrogenase, catalase, or pyruvate kinase at concentrations of 0.1 mg/mL in the assay do not replace or inhibit the trifunctional protein in the reactivation process. The latter may be viewed as an equilibrium of unknown stoichiometry governed by the dissociation constant (K_d) according to the equation

$$N_1(\text{GAR TFase}) + N_2(\text{trifunctional protein}) \underset{}{\overset{K_d}{\rightleftharpoons}} (\text{GAR TFase})_{N_1}(\text{trifunctional protein})_{N_2}$$

where

$$K_d = \frac{(\text{GAR TFase})^{N_1}(\text{trifunctional protein})^{N_2}}{[(\text{GAR TFase})_{N_1}(\text{trifunctional protein})_{N_2}]} \quad (1)$$

N = number of equivalents. Moreover, the initial velocity for GAR formation is presumed to be linearly proportional to the associated proteins, i.e.

$$v = k[(\text{GAR TFase})_{N_1}(\text{trifunctional protein})_{N_2}] \quad (2)$$

Employing an interactive computer procedure, values of K_d were varied for assumed stoichiometries 1:1, 2:1, and 3:1 (N_1–N_2) for GAR TFase–trifunctional protein to calculate the $(\text{GAR TFase})_{N_1}(\text{trifunctional protein})_{N_2}$ concentration to give the best least-squares fit to the linear equation, eq 2. A minimum stoichiometry of 3:1 GAR TFase–trifunctional protein with an average $K_d = 250 \pm 150$ nM[3] and $k = 261$ min^{-1} resulted in the "best fit" with a correlation coefficient for all 32 data points of 0.985 (Figure 4). Since the GAR TFase fraction was not obtained completely free of the trifunctional protein, it was not possible to determine directly whether the former is totally inactive in the absence of trifunctional protein; however, the intercept value for Figure 4 is zero within experimental error.

If a true equilibrium is being monitored, the same equilibrium constant should be measurable from either direction of the reaction, i.e., the concentration of the active complex $[(\text{GAR TFase})_3(\text{trifunctional protein})]$ should vary according to eq 1 when the purified complex (after AICAR–Sepharose chromatography) is diluted through K_d. Further, the trans-

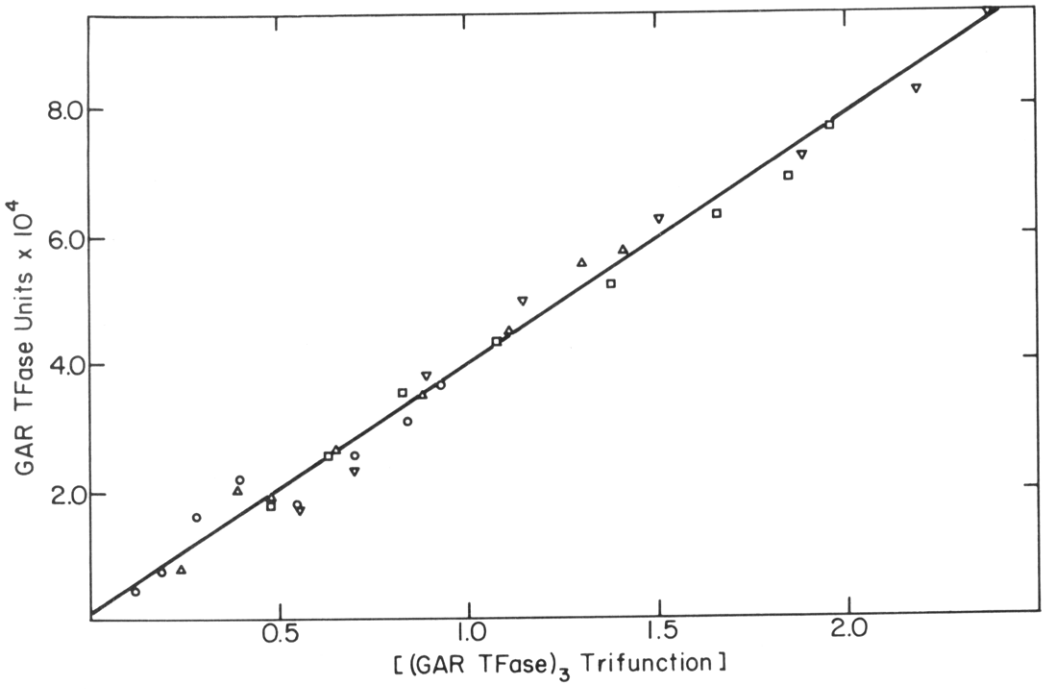


FIGURE 4: Plot of the experimental GAR TFase activity vs. calculated (GAR TFase)$_3$(trifunctional protein) concentration for K_d = 250 nM^3 and k = 261 min^{-1} according to eq 2.

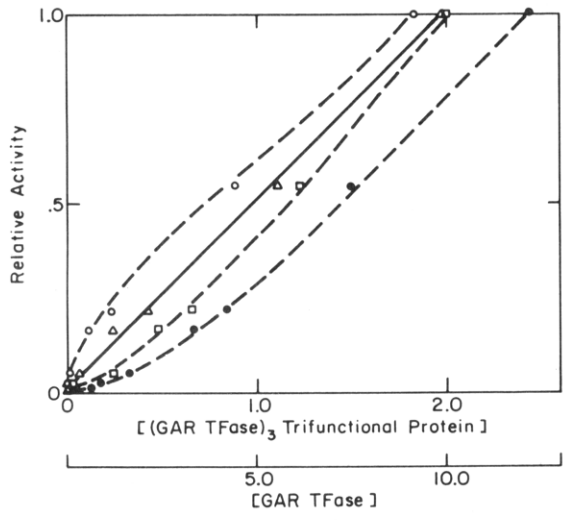


FIGURE 5: Effect of dilution upon GAR TFase activity. Plot of activity vs. GAR TFase concentration (●) and vs. (GAR TFase)$_3$(trifunctional protein) concentration when K_d = 10 (□), 150 (△), and 500 (○) nM^3. The activity was determined after the enzyme complex from the AICAR–Sepharose column had been incubated 30 min under assay conditions in the absence of 5,10-methenyl-H_4folate. The assay was started by adding the cofactor in a small volume of buffer. (Incubation of the enzyme for up to 3 h under the assay conditions in the absence of cofactor prior to assay gave identical results.)

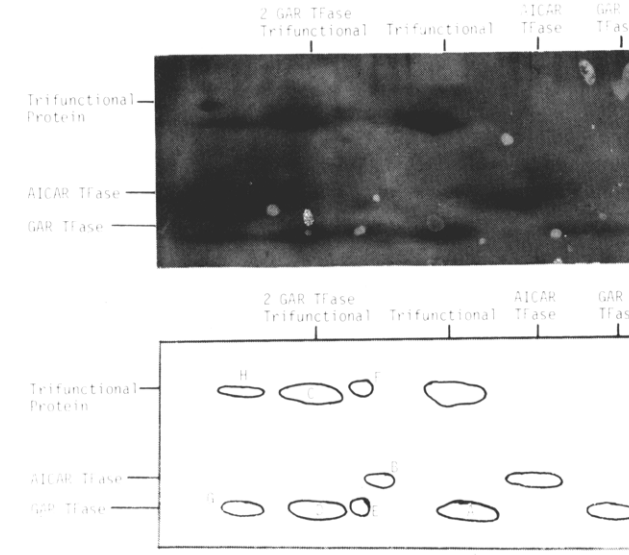


FIGURE 6: Two-dimensional NaDodSO$_4$ gel electrophoresis of cross-linked complex. The complex was cross-linked as described under Experimental Procedures. The first dimension was run in an 11-cm tube gel (5% polyacrylamide), and the cross-links then were cleaved with 2-ME. The second dimension was run by placing the tube gel on top of an 11 × 14 × 0.2 cm slab gel of 7.5% polyacrylamide.

formylase activity of the (GAR TFase)$_3$(trifunctional protein) complex should vary linearly with its calculated concentration but deviate from linearity if the value of K_d and hence the concentration of the complex are in error. The linear iterative computer program used above was modified to accept the dilution data, and K_d was varied from 1.0 to 500 nM^3 in increments of 10 nM^3. A best fit for GAR TFase activity as a function of [(GAR TFase)$_3$(trifunctional protein)] was found at $K_d \simeq 150$ nM^3 in excellent agreement with the above value. This is shown in Figure 5.

Cross-Linking. Two-dimensional NaDodSO$_4$ gel electrophoresis of proteins linked with cleavable cross-linkers has been the method of choice for the investigations of nearest-neighbor interactions in a number of protein complexes (Coggins et al., 1976; Baird & Hammes, 1976; Smith et al., 1978; Sun et al., 1974). For our study, the thiol cleavable reagent DTBP was chosen for its length (~10 Å), water solubility, and imido ester reactivity (Peters & Richards, 1977). Two-dimensional NaDodSO$_4$ gel electrophoresis was performed on the cross-linked enzyme, with the result shown in Figure 6.

In addition to the monomer spots for the trifunctional protein, AICAR TFase, and GAR TFase, four major and several minor cross-link (off-diagonal) spots are apparent. The major spots correspond to (A) GAR TFase dimer, (B) AICAR TFase dimer, (C) trifunctional protein dimer, and (C plus D) the heterologous cross-link of 2 GAR TFase units with one trifunctional protein unit. As further evidence, the minor spots for the cross-link of 1 GAR TFase unit (E) with 1 trifunctional protein unit (F) and that for 2 GAR TFase units (G) with 2

Table III: Ultracentrifugation Conditions Which Resolve the Complex Components

buffer constituents, pH 7.5	initial [protein] (mg/mL)	mL of protein
37.5 mM Tris-HCl, 30% Me_2SO, 5% glycerol, 10 mM 2-ME[a]	2.6	0.50
37.5 mM Tris-HCl, 30% Me_2SO, 5% glycerol, 10 mM 2-ME[a]	0.86	0.50
50 mM Tris-HCl, 10 mM 2-ME	0.86	0.50
10 mM K_2HPO_4, 10 mM 2-ME	2.5	0.75
50 mM Tris-HCl, 10% Me_2SO, 10 mM 2-ME	0.86	0.20
10 mM K_2HPO_4, 10 mM 2-ME[b]	25.0	0.50

[a] In the presence of glycerol, the elements of the complex did not sediment very far through the sucrose gradient; however, the activity peaks were always resolved. Ultracentrifugation was performed in 5–20% sucrose gradients containing the above constituents. [b] This experiment was performed on crude enzyme after protamine sulfate precipitation of nucleic acids to determine if any steps in the purification lead to decreased association. No increased association was seen.

Table IV: Molecular Weights for Complex Enzymes

enzyme	subunit M_r	overall M_r	no. of subunits
trifunctional	90000–97000	190000–200000	2
AICAR transformylase	66000–69000	130000–140000	2
GAR transformylase	55000–57000	110000–120000	2
Ser HMase	47000–50000	190000–215000	4

trifunctional protein units (H) are also apparent. Spots D and G are degenerate with a spot for GAR TFase tetramer and hexamer, respectively; however, cross-linking of the protein fraction containing 92% GAR TFase did not show these spots. Thus, they arise from heterologous interactions with the trifunctional protein.

The cross-linking reactions shown here were quenched with $NaDodSO_4$ rather than ammonium ion. Quenching with the latter caused the reaction solution to become turbid, and only very high molecular weight and unresolved protein bands were observed on 5% $NaDodSO_4$ gels. It is possible that the ammonium causes some association change to occur. At the protein concentration used, 0.5 mg/mL, control proteins do not cross-link.

Molecular Weights. It previously had been reported that the protein elements of this complex are resolved by sucrose density ultracentrifugation in 50 mM Tris and 10 mM 2-ME (Caperelli et al., 1980). Since we have noted that glycerol, phosphate, and Me_2SO stabilize the enzyme activities, ultracentrifugation experiments were performed in their presence. A typical sedimentation pattern is shown in Figure 7. Dissociation was observed under all the conditions reported in Table III. The subunit composition and molecular weights assigned on the basis of this study and $NaDodSO_4$ gel electrophoresis are listed in Table IV. Gel permeation chromatography on either Sephadex G-200 or Bio-Gel P-300 in the presence of stabilizers likewise resulted in partial resolution of the complex into its respective activities but did not provide reliable estimates of their molecular weights.

Sedimentation velocity and equilibrium experiments can give the overall molecular weight of a protein complex if the individual proteins do not dissociate in the centrifugal field. We have shown that dissociation does indeed occur in a sucrose gradient at 25 000 rpm. Analytical sedimentation velocity

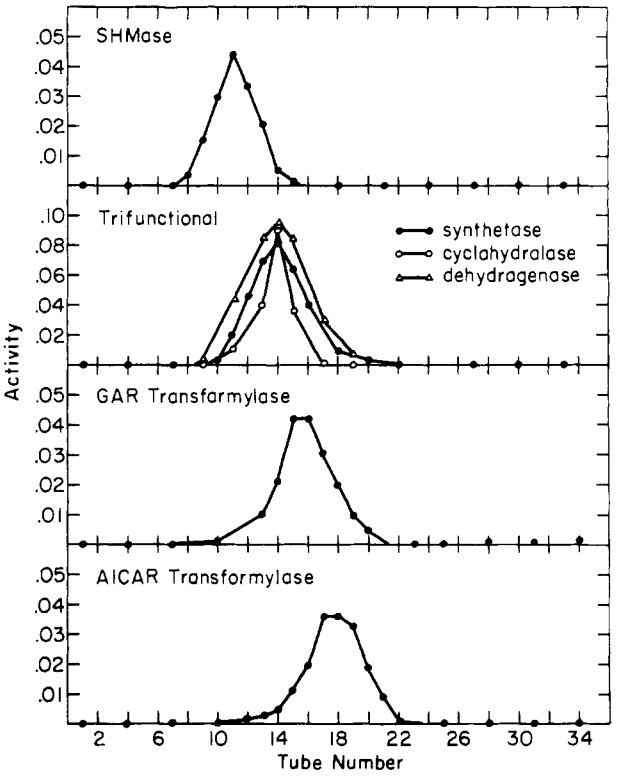


FIGURE 7: Sucrose density ultracentrifugation of complex enzymes. Protein from AICAR–Sepharose was layered onto the 5–20% sucrose gradient. Ultracentrifugation was carried out in 10 mM K_2HPO_4 and 10 mM 2-ME, pH 7.5, at 4 °C for 48 h at 25000 rpm. Upon completion, the tube was fractionated into 1-mL fractions.

Table V: Effect of Complex Concentration upon Sedimentation Coefficient in the Presence of GAR

protein concn (mg/mL)	$s_{20,w}$ (S)
0.10	6.94
0.30	7.06
0.60	7.16
0.60	7.15
1.43	7.25

experiments were performed in an attempt to determine if association would be maintained lacking a sucrose gradient. In the absence of the substrate GAR, no distinct UV boundary was formed at 0.5–2.0 mg/mL protein. Thus, lack of association is again suggested. However, in the presence of 0.25 mM (α+β)GAR, an apparent single boundary does form, suggesting that GAR causes association.

The effect of protein concentration on the sedimentation coefficient was investigated in order to determine $s_{20,w}$ extrapolated to zero protein concentration. A plot of $s_{20,w}$ vs. protein concentration gives a negative slope for nondissociating macromolecules, and the $s_{20,w}$ extrapolated to zero protein concentration is the true sedimentation coefficient (Fujita, 1962). However, a rapidly reversing associating system can lead to a plot with a positive slope (Gilbert & Gilbert, 1973; Schwert, 1949; Schachman, 1959). The sedimentation coefficients for the present system are shown in Table V. It is apparent that $s_{20,w}$ does increase with increasing protein concentration, implicating a rapidly associating and dissociating complex.

Coupling of Activities. Since the above findings were in accord with a physical interaction between the GAR TFase and the trifunctional protein, a kinetic test was undertaken of the ability of the complex to synthesize FGAR commencing

Scheme II

$$HOOC^{-}Na^{+} + ATP + H_4folate(Glu)_3 \longrightarrow$$

$$10\text{-}CHO\text{-}H_4folate(Glu)_3 \rightleftharpoons 5,10\text{-}C^{+}H\text{-}H_4folate(Glu)_3$$

$$5,10\text{-}C^{+}H\text{-}H_4folate(Glu)_3 + GAR \longrightarrow FGAR + H_4folate(Glu)_3$$

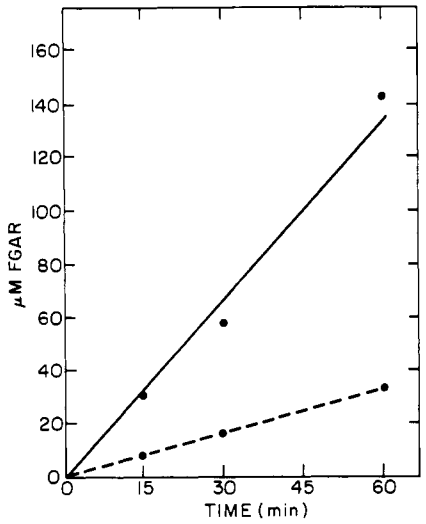


FIGURE 8: Rate of production of FGAR by the complex under kinetic coupling conditions using 14 μM H_4folate triglutamate and 2 mM sodium [^{14}C]formate (—) and from the GAR TFase assay using 2.5 μM 5,10-methenyl-H_4folate triglutamate (- - -).

with formate, ATP, and H_4folate(Glu)$_3$, thus demanding the participation of activities associated with the trifunctional protein (see Scheme II). In Figure 8 is plotted the linear rate of FGAR synthesis upon initiating its formation from formate relative to 5,10-$C^{+}H$-H_4folate(Glu)$_3$. The former pathway is 4 times more efficient than that beginning with exogenous 5,10-$C^{+}H$-H_4-folate(Glu)$_3$, the latter concentration set at 2.5 μM based on the steady-state level attained by this cofactor species during the course of FGAR synthesis (Figure 9). Neither ATP nor formate activates GAR TFase under these conditions. The ratio of 10-formyl-H_4folate(Glu)$_3$ to 5,10-$C^{+}H$-folate in solution is 4.1:1, approaching the 7:1 calculated for pH 6.8 from the reported equilibrium constant (Poe & Benkovic, 1980).

In order to test further the kinetic coupling as well as the activation upon recombination involving the trifunctional protein and GAR TFase, an iodoacetamide-modified trifunctional protein was obtained which had no measurable synthetase activity and only very low levels of cyclohydrolase and dehydrogenase activity. The effect of the iodoacetamide modification on GAR TFase activity in the recombination experiment is summarized in Table VI. Neither the modified nor unmodified trifunctional protein showed any significant GAR TFase activity when assayed separately. At concentrations twofold greater than that saturating with the unmodified protein, inactive trifunctional protein does not activate GAR TFase. A control experiment in which GAR TFase was assayed in the presence of both modified and native trifunctional protein shows that reactive ligands possibly carried over from the modification reaction are not responsible for the lack of GAR TFase activation when assayed with the modified trifunctional protein.

Discussion

The copurification of GAR TFase, AICAR TFase, Ser HMase, and the trifunctional protein possessing 5,10-methenyl-H_4folate cyclohydrolase, 5,10-methylene-H_4folate dehydrogenase, and 10-formyl-H_4folate synthetase activities

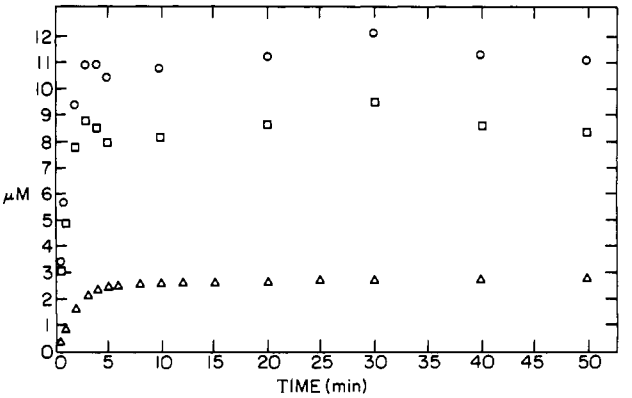


FIGURE 9: Concentrations of H_4folate cofactors produced by the complex under kinetic coupling conditions from 14 μM H_4folate triglutamate and 2 mM sodium formate: (○) 10-formyl- plus 5,10-methenyl-H_4folate triglutamate measured by ΔA_{350} after acidification; (△) 5,10-methenyl-H_4folate triglutamate measured by ΔA_{355}; (□) 10-formyl-H_4folate triglutamate determined by the difference between the total of 10-formyl- plus 5,10-methenyl-H_4folate triglutamate and 5,10-methenyl-H_4folate triglutamate alone.

Table VI: Recombination Experiment with Modified Trifunctional Protein

[GAR TFase] (nM)	iodoacetamide-modified [trifunctional protein] (nM)	native [trifunctional protein] (nM)	rel act.
12	0	0	0.22
12	26	0	0.27
12	0	12	1.0
12	26	12	1.1

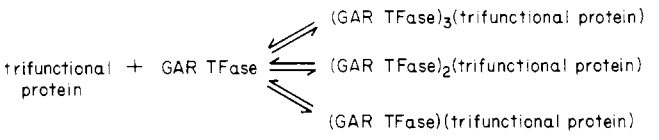
has been previously reported (Caperelli et al., 1980). The suggestion of an association between the enzymes is further supported by the results presented here. The utility of such an enzyme complex is apparent, since under complexing conditions the unstable H_4folate cofactors may not dissociate into solution prior to their use by the GAR TFase or AICAR TFase. Furthermore, the complex may furnish a convenient means for regulation of de novo purine biosynthesis.

Direct evidence for the interaction between the enzymes of this complex can be seen in the recombination, dilution, cross-linking, sedimentation velocity, and kinetic coupling experiments. The recombination and dilution experiments showed that the trifunctional protein affects a necessary and specific activation of GAR TFase. On the basis of the ordinate intercept of zero in the plot of GAR TFase activity vs. complex concentration, GAR TFase would be inactive in the absence of the trifunctional protein.

This activation must be due to a direct physical interaction between the proteins. The cofactor for GAR TFase, 5,10-methenyl-H_4folate is unstable under the assay conditions of pH 7.0, 50 mM potassium maleate, hydrolyzing with a $t_{1/2}$ of 30 min (P. A. Benkovic, unpublished data) to 10-formyl-H_4folate which is not used by the enzyme (Hartman & Buchanan, 1959). This hydrolysis is also catalyzed by the 5,10-methenyl-H_4folate cyclohydrolase activity of the trifunctional protein, and the specific activity of this enzyme is typically 10 times that of the GAR TFase. Thus, any indirect affect of the presence of the trifunctional protein should be to inhibit the GAR TFase reaction by removing cofactor. Obviously this is not the observed effect, since the enzyme indeed activates GAR TFase.

The equilibrium for this interaction is rapidly established as demonstrated by the reactivation and dilution experiments. It was found that the GAR TFase is completely reactivated

Scheme III



by a 10-min preincubation (the experimental dead time) with the trifunctional protein prior to commencing the assay with 5,10-methenyl-H_4folate. Similarly, the dilution experiments indicated that the equilibrium in the direction of dissociation is established within 30 min. Furthermore, the specificity of this recombination was demonstrated clearly through the lack of activation with several nonspecific proteins and inactivated trifunctional protein. That the latter does not affect activation additionally may indicate that processing of the cofactor species at sites on the trifunctional protein may be important. This will be the subject of a future publication.

The reversible cross-linker DTBP introduced chemical links between GAR TFase and the trifunctional protein, indicating direct interaction between the two enzymes. Thus, the cross-linking experiments are in accord with the reactivation of GAR TFase by the trifunctional protein which indicated that the two enzymes should be nearest neighbors. The major heterologous cross-link was that of two GAR TFase subunits with one trifunctional protein subunit. The amount of protein in the spot corresponding to this cross-link is comparable to that in the GAR TFase dimer spot, suggesting that the enzymes interact strongly at 0.5 mg/mL. This is an anticipated result based on the recombination dissociation constant of 250 mM^3 (or 6.3 mM for the 1:1 interaction) so that association must be >90% at 0.5 mg/mL.

The apparent lack of cross-links between AICAR TFase and either GAR TFase or the trifunctional protein should not as yet be interpreted to rule out an association between this transformylase and other proteins in the complex. Specifically, the AICAR TFase mole ratio to GAR TFase was ~0.05 in this experiment; thus, cross-linking involving AICAR TFase might not be seen. The cited cross-linking reactions were performed at pH 8.2, and it is not clear how this alkaline pH affects the protein association. Thus, at lower pH more extensive cross-linking may have been observed, although such experiments are precluded owing to more rapid imido ester hydrolysis (Hunter & Ludwig, 1962).

Although the cross-linking and recombination experiments show that GAR TFase and the trifunctional protein do indeed interact, their association must be rapidly reversible. This is supported by the sedimentation velocity, sucrose density sedimentation, and gel filtration experiments. In particular, velocity sedimentation of a system featuring rapidly reversible association between macromolecules leads to an increasing sedimentation coefficient with increasing protein concentration in dilute solution (Gilbert & Gilbert, 1973; Schachman, 1959). This characteristic presumably results from the larger population of aggregated species at higher protein concentrations due to mass action, and thus, measurements on this type of system which sediments as the weight average yield a higher apparent sedimentation coefficient with increased protein concentration (Gilbert, 1963; Schachman, 1959). This is the effect seen with the present case in accord with rapid association–dissociation. Thus, an overall molecular weight analysis cannot readily be performed on the data. Similar results are also apparent in both the sucrose density ultracentrifugation and gel filtration experiments. Here anomalous elution or incomplete resolution indicates the lack of a stable association.

The available data can be related to the outline in Scheme III. This model is supported by the observations that (1) GAR TFase is activated by 1, 2/3, or 1/3 trifunctional proteins, (2) cross-linking experiments reveal the presence of 1:1, 2:1, and 2:2 GAR TFase monomer(s)/trifunctional protein monomer(s), and (3) sedimentation analysis indicates rapid reversibility. It is important to realize that for catalytic activity only one GAR TFase need be associated with the trifunctional protein at a given time. This ratio may be sufficient to produce a more efficient FGAR synthesis as noted in experiments commencing with formate, ATP, and H_4folate$(Glu)_3$ or to result in the inability of iodoacetamide-modified trifunctional protein to activate GAR TFase.

Other examples of enzymes which only complex under specific conditions are known. The enzyme complex involved in the early steps of de novo pyrimidine biosynthesis shows tight association only in the presence of Me_2SO and glycerol (Mori & Tatibana, 1978; Coleman et al., 1977; Mori et al., 1975). In the absence of Me_2SO and glycerol the large complex of ~870 000 dissociates into smaller units. Similarly, the interaction of aspartate aminotransferase (AAT) and malate dehydrogenase (MDH) can only be demonstrated under specific conditions. Physical evidence for the complex is obtained from countercurrent distribution in 6.4% dextran, 6.6% trimethylaminopoly(ethylene glycol), or carboxymethylpoly(ethylene glycol) (Backman & Johansson, 1976). In the absence of these constituents, kinetic evidence for the interaction is observed but physical evidence is not. Indeed, chromatography of MDH on a Sephadex G-200 column equilibrated with AAT caused no anomalous elution of the MDH (Bryce et al., 1976). Halper & Srere (1977) have found that MDH and citrate synthase also show specific association in the presence of poly(ethylene glycol) whereas no apparent interaction exists in its absence. Of particular interest is the recent discovery of a tetrahydrofolate synthesizing multienzyme complex in *Escherichia coli* (Toth-Martinez et al., 1975). It would be important to determine if such a complex exists in eukaryotes and if this system interacts with the purine complex.

Acknowledgments

We thank Brian Cunningham for writing the original version of the computer program.

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Methotrexate-Resistant Chinese Hamster Ovary Cells Contain a Dihydrofolate Reductase with an Altered Affinity for Methotrexate†

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ABSTRACT: Previous reports [Flintoff, W. F., Davidson, S. V., & Siminovitch, L. (1976) *Somatic Cell Genet. 2*, 245–261; Gupta, R. S., Flintoff, W. F., & Siminovitch, L. (1977) *Can. J. Biochem. 55*, 445–452] described a series of Chinese hamster ovary cells that were resistant to the cytotoxic action of methotrexate and contained a dihydrofolate reductase that was less sensitive to inhibition by the drug than wild-type enzyme. In this study, binding of labeled methotrexate to the reductase–NADPH complex and separation of free and bound drug by filtration through Sephadex G-25 have been used to demonstrate that clonal isolates of these resistant cells contain a dihydrofolate reductase varying between 2.5- and 6-fold lower in affinity for the drug than the wild-type enzyme. The apparent dissociation constant for the wild-type enzyme is 0.5×10^{-9} M. Using two-dimensional polyacrylamide gel electrophoresis, 11 independently selected resistant isolates have been shown to contain a reductase with a similar overall net charge as the wild-type enzyme. Reductase purified from either wild-type or resistant cells contains two components after isoelectric focusing in polyacrylamide gels. The major component represents about 90% of the total protein and has a p*I* of about 8.0. The minor component representing about 10% of the reductase protein has a p*I* between 7.2 and 7.6.

Previous reports from this laboratory have described a series of Chinese hamster ovary cells (CHO)[1] that have been selected for resistance to the folic acid analogue methotrexate (Mtx) (Flintoff et al., 1976a,b; Gupta et al., 1977). Resistance in class I cells is apparently due to a structural alteration in dihydrofolate reductase, whereas the resistance in class II cells involves a defect in the permeability to Mtx. Class III cells, which were derived from class I cells by a second-step selection in an increased concentration of drug, showed increased levels of the enzyme found in class I cells.

The conclusion that class I cells are resistant because of a structural alteration in the reductase was based on the increased resistance to Mtx inhibition shown by the reductase

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[1] Abbreviations used: CHO, Chinese hamster ovary; DFBS, dialyzed fetal bovine serum; Me_2SO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; HPRT, hypoxanthine phosphoribosyltransferase; IF, isoelectric focusing; K_d, apparent dissociation constant; Mtx, methotrexate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PPO, 2,5-diphenyloxazole.

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