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Structural and Mechanistic Studies on the HeLa and Chicken Liver Proteins That Catalyze Glycinamide Ribonucleotide Synthesis and Formylation and Aminoimidazole Ribonucleotide Synthesis†

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ABSTRACT: Glycinamide ribonucleotide (GAR) transformylase from HeLa cells has been purified 200-fold to apparent homogeneity with a procedure using two affinity resins. The activities glycinamide ribonucleotide synthetase and aminoimidazole ribonucleotide synthetase were found to copurify with GAR transformylase. Glycinamide ribonucleotide synthetase and GAR transformylase were separable only after exposure to chymotrypsin. Antibodies raised to pure L1210 cell GAR transformylase were able to precipitate the glycinamide ribonucleotide transformylase and GAR synthetase activities from HeLa and L1210 cells both in their native and in their proteolytically shortened forms. The compound *N*-10-(bromoacetyl)-5,8-dideazafolate was found to inhibit formylation but to leave the ATP-requiring synthetase activities intact.

The pathway of purine de novo biosynthesis was first elucidated by Buchanan and Hartman (1959); the first five steps of the pathway are shown in Scheme I. All of the enzymes in these steps have been purified to homogeneity from avian liver due to their greater abundance in avian tissues [phosphoribosylamine (PRA)[1] amidotransferase (Hartman, 1963), GAR transformylase (Caperelli et al., 1980; Young et al., 1984), FGAM synthetase (Mizobuchi & Buchanan, 1968), and GAR synthetase and AIR synthetase (Daubner et al., 1985)]. Recent work has examined the purine synthetic pathway in neoplastic mammalian tissues, in which the levels of these enzymes are elevated in comparison to normal mammalian cells (Weber et al., 1983; Jackson et al., 1979). In particular, GAR transformylase has been purified from three murine cancer cell lines (Caperelli, 1985; Daubner & Benkovic, 1985). The recent discovery by Henikoff et al. (1985) that GAR transformylase, GAR synthetase, and AIR synthetase

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[1] Abbreviations: SDS, sodium dodecyl sulfate; HBSS, Hanks' balanced salt solution; RPMI 1640, Roswell Park Memorial Institute medium 1640; DME, Dulbecco's modified Eagle's medium; PMSF, phenylmethanesulfonyl fluoride; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; DMAC, dimethylacetamide; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; PRA, phosphoribosylamine; AIR, aminoimidazole ribonucleotide; FGAM, formylglycinamidine ribonucleotide; GAR transformylase, glycinamide ribonucleotide formyltransferase; GAR synthetase, glycinamide ribonucleotide synthetase; AIR synthetase, aminoimidazole ribonucleotide synthetase; FGAR amidotransferase, formylglycinamide ribonucleotide amidotransferase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 10-CHO-H_4folate, (6*R*)-10-formyltetrahydrofolate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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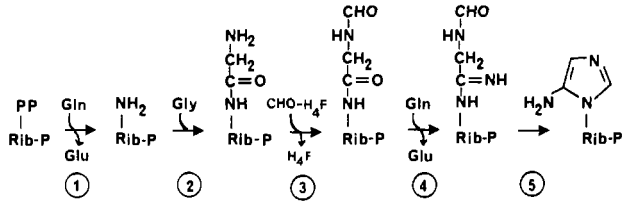
Scheme I: First Five Steps of Purine de Novo Biosynthesis[a]



[a] Enzymes are noted by the following numbers: 1, PRPP amidotransferase; 2, glycinamide ribonucleotide synthetase; 3, glycinamide ribonucleotide transformylase; 4, formylglycinamide ribonucleotide amidotransferase; 5, aminoimidazole ribonucleotide synthetase.

are encoded on overlapping polypeptides in *Drosophilia* led to the documentation of a multifunctional protein from avian liver which contains all three of these activities (Daubner et al., 1985). Patterson et al. (1982) showed that mutants of Chinese hamster ovary which lacked GAR synthetase also lacked AIR synthetase, suggesting a multifunctional protein for these steps of purine synthesis in mammals as well. Since we had previously shown (Daubner & Benkovic, 1985) that GAR transformylase levels were elevated in the order HeLa > mouse leukemia > mouse white blood cells, we set out to isolate this enzyme from HeLa and to study its structural and mechanistic properties in relation to those of the avian and murine enzymes.

Experimental Procedures

Materials

SDS gel electrophoresis marker proteins bovine serum albumin, HEPES, PMSF, ATP, D-ribose 5′-phosphate, glycine, Dowex 50W-X8, Sephadex G-200, and TPCK were obtained from Sigma Chemical Co., St. Louis, MO. [2-^{14}C]Glycine was obtained from Amersham, Arlington Heights, IL. Chymotrypsin was purchased from Worthington, Freehold, NJ. ^{125}I-Protein A was purchased from New England Nuclear, Boston, MA. Electrophoresis chemicals, protein assay kit, and Affigel Blue were obtained from Bio-Rad Laboratories, Richmond, CA. Ultrapure urea was obtained from Schwarz/Mann, Cambridge, MA. RPMI 1640, DME, HBSS, trypsin, sodium bicarbonate, and goat anti-rabbit serum were obtained from GIBCO Laboratories, Grand Island, NY. Fetal bovine serum, horse serum, penicillin, and streptomycin were purchased from Flow Laboratories, McLean, VA. Kodak XAR-5 film and Du Pont Cronex lightning plus intensifier screens were purchased from Picker International, Highland Heights, OH.

Mouse leukemia L1210 cells were the generous gift of Dr. J. Bertino of the Yale University School of Medicine. HeLa O cells were the generous gift of Dr. D. R. Tershak, The Pennsylvania State University. 10-Formyl-5,8-dideazafolate and 10-formyl-5,8-dideazafolate–Sepharose were prepared as previously described (Young et al., 1984). GAR was synthesized by the method of Chettur and Benkovic (1977). FGAM was the kind gift of Dr. JoAnn Stubbe, University of Wisconsin.

Methods

Enzyme Assays. GAR synthetase was assayed as described by Daubner et al. (1985) but using 5 mM ATP and incubating the reaction for a longer period of time. For mammalian samples, incubations ran for 1 h, and intermediate time points were taken every 20 min. GAR transformylase was assayed with a folate analogue. The rate of transfer of the formyl group from 10-formyl-5,8-dideazafolate to GAR was determined by the method of Young et al. (1984) except that the buffer used was 37.5 mM HEPES (pH 7.5)/20% glycerol. The course of this reaction was monitored at 295 nm, and $\Delta\epsilon_{295}$ = 18 900 M^{-1} cm^{-1} (Smith et al., 1981). AIR synthetase was assayed as described by Daubner et al. (1985) but using 5 mM ATP and incubating the reaction for about 30 min.

Protein determination was performed either by measuring A_{280} and using bovine serum albumin as a standard or by using the Bio-Rad protein assay with ovalbumin as a standard.

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970), employing slabs of 8.75% polyacrylamide. When standard curves for molecular weight calibration were needed, a mixture of the proteins myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase was electrophoresed in a lane adjacent to the samples of interest. Samples were denatured by boiling for 10 min in a solution of 1% SDS, 1% 2-mercaptoethanol, 50 mM Tris-HCl, pH 8.8, and 5% glycerol. Gels were routinely stained for 1 h by immersion in 0.05% Coomassie blue R-250 in 25% 2-propanol/10% acetic acid and destained overnight in 10% 2-propanol/10% acetic acid.

Western Blots. SDS gel electrophoresis was carried out according to Neville (1971) when the resulting slab gel was intended for use in Western blotting. Electrophoretic transfer of proteins from polyacrylamide to paper was performed according to Reiser and Stark (1983) with all the modifications noted by Young et al. (1984). Antibodies for probing the blots were prepared in rabbits with L1210 GAR transformylase according to the method of Crowle (1973). Aminothiophenol paper was prepared according to Seed (1982).

Cell Culture and Harvest. All cells were maintained at 37 °C under a 5% CO_2 atmosphere. Cells were counted with a hemocytometer. All media were prepared from dry powdered mixtures with double-distilled water. Tissue culture grade bicarbonate was added to media; RPMI 1640 contained 1.25 g/L, and DME contained 3.75 g/L. Cell media were sterilized by pumping through Millipore Sterivex GS filters.

HeLa O cells were maintained as monolayer cultures in Corning 850 cm^2 roller bottles in DME, 10% fetal bovine serum, 50 IU of penicillin/mL, and 50 μg of streptomycin/mL. They were harvested by trypsinization with 0.05% trypsin in 0.74 mM EDTA in Dulbecco's phosphate-buffered saline, followed by two washes in HBSS. PMSF was added from a 20 mg/mL stock in methanol in a ratio of 1 mg/10^9 cells. Cell disruption was accomplished by three freeze–thaw cycles and 25 passages through a 23-gauge needle. L1210 cells were cultured and harvested as described by Daubner and Benkovic (1985).

Enzyme Isolation. GAR transformylase was purified from L1210 cells as described by Daubner and Benkovic (1985). This purification protocol was modeled closely after the procedure of Young et al. (1984) for the isolation of the chicken enzyme which consists of one affinity column step using 10-formyl-5,8-dideazafolate–Sepharose. A second step was added to the protocol for the isolation of HeLa enzyme using a Cibacron Blue linked resin marketed by Bio-Rad Laboratories as Affigel Blue. All enzyme isolation steps were performed at 4 °C.

HeLa cells were harvested and disrupted as described in the previous section. The crude homogenate was centrifuged at 31900*g* for 1 h. The supernatant was dialyzed vs. 37.5 mM potassium phosphate (pH 7.4)/20% glycerol to remove the high levels of NaCl from the saline buffer which was used to wash the cells. The dialyzed supernatant was applied to a column of 10-formyl-5,8-dideazafolate–Sepharose (3.0 × 1.5

cm). Loading was carried out at a rate of 1 mL/min. Washes were performed at a flow rate of 3 mL/min, beginning with a wash of 37.5 mM potassium phosphate (pH 7.4)/20% glycerol followed by a wash with 2 M KCl in 37.5 mM potassium phosphate (pH 7.4)/20% glycerol. Each wash consisted of 5 column volumes of buffer. Elution of the enzyme was effected with 2 M urea, 37.5 mM potassium phosphate, pH 7.4, and 20% glycerol at a rate of 0.75 mL/min. The active fractions were pooled and dialyzed vs. 37.5 mM potassium phosphate (pH 7.4)/20% glycerol.

The Affigel Blue column had dimensions of 1.0 × 6 cm. The dialyzed pool from the deazafolate-bound resin was applied and loaded at a slow rate (1 mL/min). Washes of this affinity column proceeded at 3 mL/min; the first wash was done with 37.5 mM potassium phosphate (pH 7.4)/20% glycerol, and the second was done with 1 M urea in 37.5 mM potassium phosphate (pH 7.4)/20% glycerol. Five column volumes of each buffer were passed through the column. Elution was performed with 2 M KCl in 37.5 mM potassium phosphate (pH 7.4)/20% glycerol, and the activities emerged before 10 mL had passed through the column. The active fractions were pooled, dialyzed vs. 37.5 mM potassium phosphate (pH 7.4)/20% glycerol, and concentrated by embedding the dialysis bag containing the enzyme in a mound of Sephadex G-200.

Chymotryptic Digest. Two sets of duplicate samples of the protein, one set of L1210 and the other of HeLa enzyme purified on 10-formyl-5,8-dideazafolate–Sepharose, were set up in a volume of 0.7 mL in 12.5 mM potassium phosphate, pH 7.4, 20% glycerol, and 1 mM $MgCl_2$. To one tube of each set was added chymotrypsin: 80 ng (3 pmol) was added to 100 μg (890 pmol) of L1210 protein, and 40 ng (1.5 pmol) of protease was added to about 55 μg (490 pmol) of HeLa enzyme. These samples as well as the control tubes were incubated at room temperature for 3 h, when 10.6 μg (30 pmol) of TPCK from a stock of 1.37 mg/100 mL of methanol was added to quench proteolysis. TPCK was added to control tubes as well as protease-containing tubes. The mixtures were rocked at 4 °C for 10 min. One-half milliliter of packed 10-formyl-5,8-dideazafolate–Sepharose was added to each sample, and they were gently rocked at 4 °C for 20 min. The samples were then spun in an Eppendorf centrifuge for 5 min, and the supernatants were removed. The resin was washed with 0.4 mL of 12.5 mM potassium phosphate, pH 7.4, 15% glycerol, and 1 mM $MgCl_2$ by rocking at 4 °C for 10 min, and this wash was similarly removed. Elution was performed by adding 0.6 mL of 2 M urea in the same buffer and again rocking for 20 min. This elution was repeated with 0.4 mL, and the urea-containing supernatants were pooled. All samples were assayed immediately for GAR synthetase and GAR transformylase activities, separated by SDS–polyacrylamide gel electrophoresis, and subjected to Western blotting and immunoprecipitations.

Immunoprecipitations. Enzyme preparations were mixed in a 9:1 volume ratio with a stock buffer containing 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 50 mM EDTA, and 1% Nonidet P-40. This enzyme mixture was distributed into a series of tubes to which varying amounts (usually 3–30 μL of serum to 35 μL of enzyme) of antisera and preimmune sera were added. These samples were rocked gently at 4 °C for 2 h. Next goat anti-rabbit antiserum was added, usually 75 μL per 35 μL of enzyme. Samples were rocked overnight at 4 °C. Samples were centrifuged for 5 min in an Eppendorf centrifuge, and the supernatants were assayed for GAR synthetase and GAR transformylase.

Synthesis of 10-(Bromoacetyl)-5,8-dideazafolate. Synthesis of this compound was done by using the general method of Temple et al. (1982). Aliquots of a 1 M solution of bromoacetyl bromide in $CDCl_3$ were added to a solution of 8.5 mg (18 μmol) of 5,8-dideazafolate in 0.4 mL of DMAC. The reaction was monitored by changes in its UV spectrum; typically 50–60 μL (ca. 50 μmol) was needed to complete the conversion.

When the reaction was complete, water was added until the solution became cloudy (typically 2–3 mL of H_2O). Four drops of 0.1 N NaOH were added, and the suspension was incubated on ice 1 h to allow full precipitation. The suspension was centrifuged at 4 °C, 10500 rpm (18000*g*) for 10 min, and then decanted. The off-white precipitate was washed with chilled absolute ethanol, centrifuged as before, and then dried in vacuo over P_2O_5 overnight. Occasionally upon addition of ethanol, a very fine suspension/emulsion formed. When this happened, ethanol was removed by a dry argon stream. The yield was 1–3 mg of an off-white solid, portions of which were dissolved in 20 mM potassium phosphate, pH 7.5, and stored at 4 °C.

Determination of Active Inhibitor Concentration. The assay for the bromoacetyl group is based on its lability to a R–SH nucleophile. A variant of the procedure of Bednar et al. (1982) was used. An aliquot (1–6 μL) of inhibitor at 1–5 mM was delivered to a mixture of 2 μL of ca. 10 mM glutathione/100 mM TAPS, pH 7.4, and 12 μL of 0.89 M Tris, 0.89 M borate, and 25 mM EDTA, pH 8.25. When the reaction was complete, the entire mixture was injected into a cuvette containing 1 mL of 1 mM DTNB, 10 mM EDTA, and 90 mM potassium phosphate, pH 7.0. Free thiol concentration was quantitated by using ϵ_{412} = 13600 M^{-1} cm^{-1} (Ellman, 1959). The difference between this concentration and an identical control (replacing inhibitor solution by 20 mM potassium phosphate, pH 7.5) gave the concentration of R–SH-labile groups. A time study on this reaction indicated that it was complete after 20 min.

Inactivation Kinetics. Inactivation kinetics with 10-(bromoacetyl)-5,8-dideazafolate were done by diluting 5 μL of the enzyme solution into 1.0 mL of 20 mM HEPES, pH 7.5, at 25 °C, making the enzyme concentrations about 40 nM for chicken and about 2 nM for mammalian enzyme. A zero time, assay was done before adding aliquots of inhibitor. After addition of 10-(bromoacetyl)-5,8-dideazafolate, 50-μL portions of the incubation mixture were withdrawn at the specified times and assayed for enzyme activity.

Results and Discussion

A summary for the isolation of HeLa enzyme appears in Table I. The one-step procedure used for purifying chicken and L1210 enzymes exhibiting GAR transformylase activity did not yield pure protein from HeLa; the enzyme was only about 10% pure on SDS gels. An Affigel Blue column was added since 1 M urea would not release GAR transformylase activity from Cibacron Blue resins. This two-step procedure resulted in enzyme judged to be about 80–99% pure by SDS gels, with an overall yield of 44%. An SDS gel of the purified HeLa enzyme is shown in Figure 1. As demonstrated previously for chicken liver (Daubner et al., 1985), two other activities of purine de novo biosynthesis, GAR synthetase and AIR synthetase, copurify with GAR transformylase, as seen by the constant ratios of the three specific activities recorded in Table I. At no step during this purification procedure were the enzyme activities separated to any degree. Similar final ratios were seen for the purified L1210 and chicken enzymes (last entries, Table I). We did not assay for the intervening

Table I: Copurification of GAR Transformylase, GAR Synthetase, and AIR Synthetase Activities from HeLa Cells[a]

step	mg of protein[b]	sp act. [nmol of product/(min·mg)] AS[c]	GS	GT	sp act. ratio, AS:GS:GT	x-fold purification[d]	% yield[e]
HeLa, homogenization supernatant	386	0.125	1.09	1.86	1:8.8:15		100
dideazafolate, resin eluate	4.08	12.6	77.8	163	1:6.2:13	87	91
Affigel Blue eluate	0.80	24.0	228	431	1:9.5:18	210	44
chicken					1:4.4:4.8		
L1210					1:3.8:13		

[a] Number of cells harvested for this enzyme preparation was 1.55×10^9. Cells were harvested at confluence. [b] Data obtained with the Bio-Rad protein assay. [c] AS = AIR synthetase, GS = GAR synthetase, and GT = GAR transformylase. [d] Obtained by averaging the purification factors for the three activities. [e] Obtained by averaging the yield for the three activities.

Table II: Separation of GAR Transformylase and GAR Synthetase by Chymotryptic Cleavage and Immunoprecipitation of Separated Activities

			act. remaining after immunoprecipitation[a] preimmune serum		immune serum	
	GS	GT	GS	GT	GS	GT
L1210, starting enzyme	1.1[b]	15.2	0.98	13.7	0.10	0.91
L1210, control urea eluate	1.2	8.5	nd[c]	nd	nd	nd
L1210 clip						
supernatant	0.46	0.76	0.42	0.67	0.037	0.045
KP_i wash	0.30	0.18	nd	nd	nd	nd
urea eluate	0.54	5.7	0.49	7.9	0.043	0.51

[a] Activity in supernatant. [b] Activities expressed in nanomoles of product per minute per milliliter. Volumes are the following: starting enzyme, 0.7 mL; supernatant, 0.7 mL; KP_i wash, 0.4 mL; urea eluate, 1.0 mL. [c] nd = not determined.

FIGURE 1: SDS gel electrophoresis analysis of purified HeLa GAR transformylase. The standards, in the left lane, are β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase. The lane on the right contains 8 μg of HeLa GAR transformylase purified by the two-column procedure described under Methods.

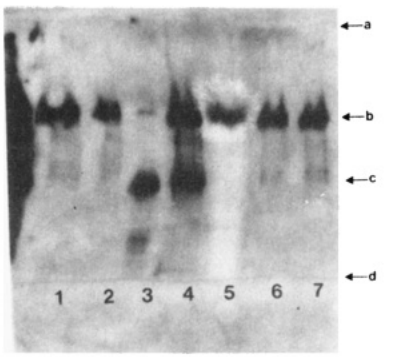


FIGURE 2: Western blot of L1210 GAR transformylase samples from the chymotryptic digest and HeLa GAR transformylase samples from the isolation protocol. Lane 1 contains L1210 enzyme which was used for the digest experiment; lane 2 is from the control for that experiment, and lanes 3 and 4 contain the samples from the actual digest. Lanes 5–7 contain HeLa samples. The contents of each lane are as follows: lane 1, L1210 GAR transformylase, 30 μg of protein; lane 2, control sample urea eluate, 10 μg of protein; lane 3, digest sample supernatant, 10 μg of protein; lane 4, digest sample urea eluate, 17 μg of protein. The HeLa samples shown are as follows: lane 5, HeLa homogenization supernatant, 2.6 mg; lane 6, HeLa 10-formyl-5,8-dideazafolate–Sepharose eluate, 100 μg; lane 7, HeLa Affigel Blue eluate, 10 μg. The letters designate the following positions on the Western blot: (a) top of gel; (b) $M_r \simeq 110000$; (c) $M_r \simeq 55000$; (d) bromophenol blue front. The blot was probed with 75 μL of crude rabbit serum to L1210 GAR transformylase and 1 μCi of ^{125}I-protein A.

activity, FGAR amidotransferase, since in chicken liver it is not a property of the same polypeptide chain as the other three activities (Daubner et al., 1985). The subunit molecular weight of the HeLa enzyme, estimated at 112000 (Figure 1), is compared to the same value for the L1210 enzyme and 110000 for the chicken liver enzyme (Young et al., 1984).

In view of the possibility that minor contaminants of the mammalian preparations were responsible for GAR synthesis and AIR synthesis, immunoprecipitations were performed with antibody which was raised in rabbits after injections of the L1210 enzyme which had been judged homogeneous by SDS gel electrophoresis. Such antisera were seen to precipitate 90–95% of the GAR transformylase and GAR synthetase activities in crude HeLa and L1210 cell extracts as well as in purified samples. These antibodies identified one major band in Western blots of crude samples of HeLa and L1210 and in purified samples (data for HeLa shown in Figure 2), in support of the hypothesis that GAR transformylase and GAR synthetase activities belong to the same polypeptide chain.

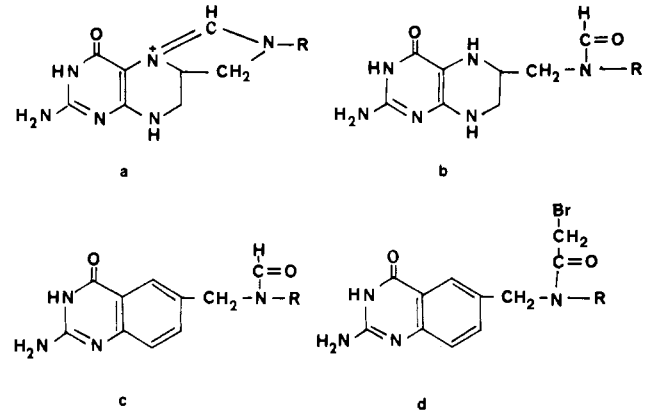
Partial digestion of the chicken and L1210 enzymes by chymotrypsin has shown that they are degraded to a smaller form during the first 3 h of digestion, during which the GAR transformylase activity remained intact as compared to controls (Young et al., 1984; Daubner & Benkovic, 1985). Further digestion resulted in loss of activity. It was also demonstrated for the avian enzyme that the initial clip by chymotrypsin generates two polypeptides: one that does not bind to 10-formyl-5,8-dideazafolate–Sepharose and possesses GAR synthetase activity and one that binds to the resin and contains GAR transformylase activity. The documentation of a similar

attempt to separate these activities on the proteins obtained from L1210 cells by proteolysis followed by affinity chromatography appears in Table II. The second and third columns record the activities in each fraction. The control was a sample which was incubated at 25 °C in the absence of chymotrypsin and then subjected to chromatography on 10-formyl-5,8-dideazafolate–Sepharose. In the control, all the recoverable activities were found in the 2 M urea eluate. However, after proteolysis, considerable GAR synthetase activity was found in the supernatant and wash fractions, while GAR transformylase activity appeared to the greatest extent in the urea eluate. Presumably, the chymotryptic clip was not complete, explaining why some GAR synthetase activity was able to bind to the resin until release by 2 M urea. The sample then was dialyzed vs. 25 mM potassium phosphate (pH 7.4)/20% glycerol to remove urea. It was subjected to immunoprecipitation with antibody to the L1210 enzyme, and the supernatant was assayed for GAR synthetase and GAR transformylase activities. These data appear in the fourth through seventh columns of Table II and show that GAR synthetase activity released after chymotrypsin treatment is still precipitable by antibody that complexes GAR transformylase activity, presumably since the GAR synthetase domain of the protein was a part of the original antigen.

A Western blot was performed in order to determine the molecular weight of the fragment responsible for GAR synthetase activity. The above antibody to the L1210 enzyme identified only one band of M_r 110 000 in the SDS gel electrophoresis of either crude extracts or purified L1210 and HeLa proteins (Figure 2, lanes 1 and 5). If after chymotryptic digestion of those samples a lower molecular weight band could be detected by the same antibody, the result would be consistent with cleavage of a single protein into two fragments, one catalyzing GAR synthesis and the other GAR formylation. The Western blot shows a smaller molecular weight band at M_r 55 000 (lanes 3 and 4) in chymotrypsin-treated samples of the L1210 enzyme estimated by comparison to fragments from the avian protein. The fragment in the supernatant fraction (lane 3) is associated with GAR synthetase activity and that in the urea eluate fraction (lane 4) with GAR transformylase activity. For all three forms of the enzyme (avian, murine, and human), the protein segment possessing GAR transformylase activity is more sensitive to additional proteolytic breakdown, since its recovery is never as complete as recovery of GAR synthetase activity. In particular, similar experiments on the HeLa protein resulted in the loss of greater than 65% of GAR transformylase activity by the third 0.5 h of the control incubation. This instability coupled with low available levels of HeLa enzyme precluded a successful Western blot experiment.

Steady-State Kinetics. Steady-state kinetic parameters for the HeLa enzyme were determined for comparison to the avian and murine forms. Assays were done in HEPES instead of phosphate since the latter is an inhibitor causing the $K_{M,app}$ for GAR to increase 5-fold (S. C. Daubner, unpublished results). The K_M for GAR is 9 μM (compared with 45 and 90 μM for chicken and L1210, respectively), the K_M for the folate analogue 10-formyl-5,8-dideazafolate is 2 μM, which is quite similar to the values for chicken and L1210 GAR transformylase. The turnover number for HeLa GAR transformylase with this cofactor is 900/min, which is comparable to the chicken enzyme and about 4-fold faster than the L1210 enzyme. It is important to mention that, as shown previously for the chicken and murine enzymes, the physiological substrate is 10-CHO-H_4folate (and not 5,10-methenyltetrahydrofolate), which has a K_M of about 10 μM. The structures of these compounds are shown in Scheme II.

Scheme II: Chemical Structures of (a) 5,10-Methenyltetrahydrofolate, (b) 10-Formyltetrahydrofolate, (c) 10-Formyl-5,8-dideazafolate, and (d) 10-(Bromoacetyl)-5,8-dideazafolate



Properties of 10-(Bromoacetyl)-5,8-dideazafolate. In order to study the mechanism of the reaction carried out by GAR transformylase and as further probe of the physical arrangement of the three catalytic activities on the peptide chain, we wished to devise a site-specific inhibitor of the enzyme. Since aminolysis of amides is both acid and base catalyzed (Jencks, 1969) and aminolysis of model compounds for C^1-tetrahydrofolates at the formate oxidation level has been shown to be general base catalyzed (Bullard et al., 1974), the active site might be imagined to be sensitive to alkylating agents. An affinity label that has been used with success because of its ability to react with amino acid nucleophiles is the bromoacetyl group (Wilchek & Girol, 1977). The methodology of Temple et al. (1982) for the synthesis of 10-(chloroacetyl)folic acid was used to derivatize the cofactor analogue 5,8-dideazafolate because of its greater stability in air. The structure of the inhibitor is shown in Scheme II, along with the natural folate substrate 10-formyltetrahydrofolate and the quinazoline analogue 10-formyl-5,8-dideazafolate.

The inhibitor was analyzed by NMR and mass spectroscopy. The presence of the CH_2Br resonance at 3.9 ppm in proper proportion to the quinazoline aromatic proton resonances indicated the incorporation of one bromoacetyl group per dideazafolate. The molecular ion peak in the mass spectrogram at m/e 562 showing a characteristic bromine isotopic pattern indicated the presence of one bromine atom in the molecule. The UV spectral change for bromoacetylation is similar to that obtained for formylation of the starting material and appears to be general for substitution at N-10. Using the glutathione assay for bromine equivalents already described, we determined the extinction coefficient at 310 nm to be 4190 M^{-1} cm^{-1}. Again, the overall spectral characteristics are similar to those of 10-formyl-5,8-dideazafolate, which has ϵ_{310} = 3800 M^{-1} cm^{-1} (Smith et al., 1981). The stability of 10-(bromoacetyl)-5,8-dideazafolate was also studied by repeated assay by glutathione of a solution of the compound in 20 mM potassium phosphate, pH 7.5 at 4 °C, indicating a $t_{1/2} \simeq 3$ weeks. A time course for reaction of 10-(bromoacetyl)-5,8-dideazafolate with glutathione showed that about 20 min is needed for complete reaction. This compares with the 5 min needed for complete reaction of 3-bromo-2-ketoglutaric acid (Bednar et al., 1982). In addition, it was found necessary to react 10-(bromoacetyl)-5,8-dideazafolate with glutathione in buffers of pH 8.0 or greater; the 100 mM TAPS, pH 7.4, used by Bednar et al. was insufficient. Thus, assays done in TAPS, pH 7.4, detected

Table III: Inactivation of GAR Transformylase Activity with 10-(Bromoacetyl)-5,8-dideazafolate

GAR transformylase source	K_I[a] (μM)	k_3[a] (min^{-1})
chicken	3	0.23
L1210	1	0.09
HeLa	1	0.16

[a] ±10%.

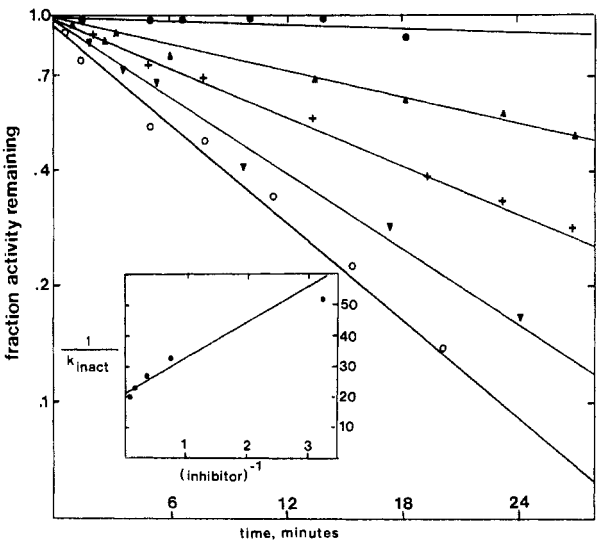


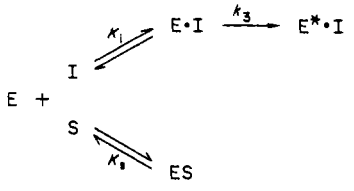
FIGURE 3: Plot of log percent activity remaining vs. time for various concentrations of 10-(bromoacetyl)-5,8-dideazafolate. Inhibitor was added to 0.0371 μM enzyme, and aliquots were assayed as described under Methods. The symbols referred to the following inhibitor concentrations: closed circles, no inhibitor; upright triangles, 0.31 μM; plus signs, 1.37 μM; inverted triangles, 2.7 μM; open circles, 10.3 μM. The inset shows a replot of the reciprocal of the slope vs. the reciprocal of the inhibitor concentration.

only 50% of the labile bromoacetyl groups in solution.

Kinetics of Enzyme Inactivation. The 10-(bromoacetyl)-5,8-dideazafolate was a good inhibitor of all three GAR transformylases (see Table III). All were inhibited in a time-dependent fashion according to the model:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E^*{\cdot}I$$

where E*·I is inactivated enzyme. When the kinetics of inactivation were analyzed as log percent activity remaining vs. time (plot for L1210 enzyme shown in Figure 3) according to Strickland et al. (1975), the values for k_3 noted in Table III were obtained. More extensive kinetic studies were done with the chicken liver GAR transformylase because of the greater availability of this enzyme. 5,8-Dideazafolate protects against inactivation by 10-(bromoacetyl)-5,8-dideazafolate in accordance with the scheme:



where $1/\text{slope} = 1/k_3 + (1 + [S]/K_s)K_i/k_3[I]$. Figure 4 shows the results in the form of the primary replot of 1/slope vs. 1/[I]. The data fit the above scheme, indicating that 10-(bromoacetyl)-5,8-dideazafolate competes with 5,8-dideazafolate and thus is active site directed. The parameters (±10%) obtained are K_I = 1.53 μM, K_s = 2.09 μM, and k_3 = 0.37 min^{-1}, in satisfactory agreement with those listed in Table III.

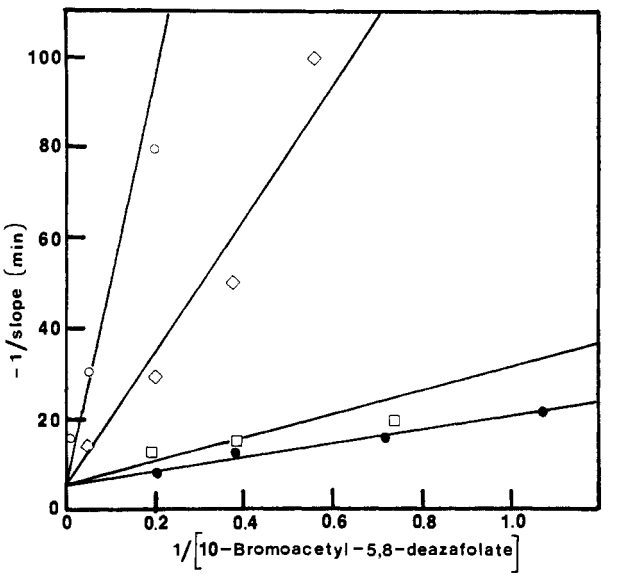


FIGURE 4: Primary replot of the reciprocal of the slope vs. the reciprocal of 10-(bromoacetyl)-5,8-deazafolate concentration at various concentrations of added 5,8-dideazafolate. Assays were done as described for Figure 3: closed circles, 0.77 μM; open squares, 4.28 μM; open diamonds, 20.8 μM; open circles, 40.8 μM 5,8-dideazafolate.

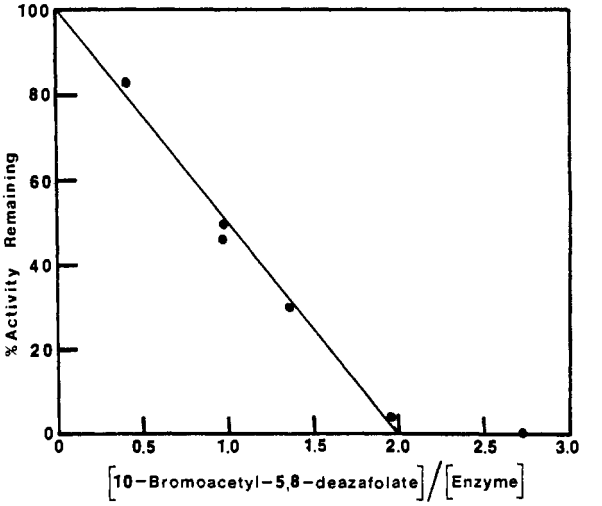


FIGURE 5: Stoichiometry of inactivation of GAR transformylase by 10-(bromoacetyl)-5,8-dideazafolate. Aliquots of inhibitor were incubated up to 1.5 h with 8.3 μM GAR transformylase (20 μM HEPES, pH 7.5, 25 °C) as described under Methods. The reaction mixture was assayed until no further inactivation was seen, and the remaining activity was plotted vs. the amount of inhibitor added.

Stoichiometry of Inactivation. Figure 5 shows the results of a titration of 10-(bromoacetyl)-5,8-dideazafolate vs. enzyme activity. Aliquots of enzyme were incubated with different concentrations of inhibitor, and the activity remaining after inactivation had ceased was measured. As shown, a clean titration was obtained, giving 2 bromoacetyl equiv per enzyme monomer required for complete loss of activity. This result was obtained by using the avian enzyme, and a similar number was obtained when the titration was repeated with HeLa enzyme. However, it would be premature to interpret the titration result solely in terms of two transformylase sites per monomer.

When the enzymes from avian liver and from HeLa cells were incubated with 5 equiv of 10-(bromoacetyl)-5,8-dideazafolate at 0 °C for 60 min, less than 5% of the GAR transformylase activity remained, while 100% of GAR synthetase, 75% of HeLa AIR synthetase, and 63% of chicken AIR synthetase remained intact. This, along with the data

showing that 5,8-dideazafolate inhibits inactivation by 10-(bromoacetyl)-5,8-dideazafolate, suggests that the inhibitor acts primarily at the folate site, further supporting the hypothesis inferred from the proteolysis experiments that the active sites are separate. In conclusion, this study describes the first isolation of these three activities of purine de novo biosynthesis from a human source. The three appear to reside on a single polypeptide isolated from avian, murine, and human cell lines. The possibility that the three activities copurify on three distinct proteins of identical molecular weight cannot be discounted by immunoprecipitation with polyclonal antibodies. Nevertheless, the studies reported herein coupled with extensive work on the avian species, that includes three unrelated purification schemes yielding the same trifunctional protein (Daubner et al., 1986), collectively support a single enzyme species for all three sources.

Registry No. GAR, 10074-18-7; GAR transformylase, 9032-02-4; GAR synthetase, 9032-01-3; AIR synthetase, 9023-53-4; 10-CHO-H_4folate, 74644-66-9; 10-(bromoacetyl)-5,8-dideazafolate, 101375-70-6; bromoacetyl bromide, 598-21-0; 5,8-dideazafolate, 5854-11-5; 10-formyl-5,8-dideazafolate, 61038-31-1.

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