Glycyl-tRNA Synthetase from Baker's Yeast. Interconversion between Active and Inactive Forms of the Enzyme†

Daniel Kern,* Richard Giegé, and Jean-Pierre Ebel

ABSTRACT: Glycyl-tRNA synthetase from baker's yeast has been purified to homogeneity. This synthetase was found to be very sensitive to proteases present in the yeast extracts and to oxidizing agents of thiol groups. In the absence of protease inhibitors and/or dithioerythritol, the enzyme rapidly lost its activity and could not be isolated. The use of these protectors allowed us to obtain different oligomeric structures of the synthetase. In the presence of a minimal concentration of dithioerythritol but in the absence of protease inhibitors, a tetrameric glycyl-tRNA synthetase of the $\alpha_2\beta_2$ type (α = 67 600, β = 57 500) with a very low specific activity was recovered. With high concentrations of both protectors, a dimeric enzyme was isolated with a specific activity comparable to that found for other yeast synthetases. The enzyme was of the α_2 type where α = 70 000–80 000 daltons, depending on whether phenylmethanesulfonyl fluoride or diisopropyl fluorophosphate was used as the protecting agent. The native form of the enzyme (α_2 = 160 000) associated easily with other proteins in various complexes of molecular weights from 250 000 to 300 000, some of them containing valyl-tRNA synthetase. The dimeric glycyl-tRNA synthetase was found in equilibrium with its subunits. Diluting the enzyme solution or increasing the salt concentration displaced the equilibrium toward the monomers, which are catalytically inactive for both the tRNA aminoacylation and the PP_i–ATP exchange reactions. Addition of both $tRNA^{Gly}$ and ATP·$MgCl_2$ plus glycine displaced the equilibrium toward the dimeric form of the enzyme. Thiol groups were found to be involved in the association between the two subunits and in both activities of the synthetase. The results are interpreted in the light of possible regulatory mechanisms of the activity of this synthetase.

The aminoacyl-tRNA synthetases exhibit a considerable diversity in their structural and catalytic properties. Monomeric (α type) and polymeric (α_2 and $\alpha_2\beta_2$ types) structures have been described [for a general review, see Schimmel & Söll (1979)]. The significance of this wide variety of subunit sizes and oligomeric structures remains unclear. Because proteases have been detected in the crude extracts of various organisms (Pringle, 1975; Kern et al., 1975, 1977; Kellermann et al., 1979), the subunit structure of some synthetases could result from the polymerization of proteolytic fragments arising from monomeric native structures. However, since oligomeric structures were often purified in the presence of protease inhibitors, it can be assumed that some, if not all, are native species.

The glycyl-tRNA synthetases so far studied were obtained from prokaryotic organisms. An $\alpha_2\beta_2$ structure has been reported for the enzymes from *Escherichia coli* (Ostrem & Berg, 1974) and *Bacillus brevis* (Surguchov & Surguchova, 1975), whereas an α_2 dimer was found for the enzyme from *Bacillus stearothermophilus* (Grosjean et al., 1976). No information is available concerning this aminoacyl-tRNA synthetase in eukaryotic cells.

We report here the isolation and the structure of glycyl-tRNA synthetase from baker's yeast. Purification of this enzyme was difficult because of its high sensitivity to oxidizing agents and to the proteases present in yeast extracts. Different enzyme forms varying in their structural properties and specific activities were obtained whether the purification was performed in the absence or in the presence of protease inhibitors. Under nonoptimal protecting conditions, an $\alpha_2\beta_2$ structure was isolated, whereas in the presence of diisopropyl fluorophosphate (DIFP)[1] or phenylmethanesulfonyl fluoride (PMSF)[1] a putative native form of the α_2 types was obtained. This dimeric glycyl-tRNA synthetase can tightly associate with other proteins, for instance with valyl-tRNA synthetase. It is in equilibrium with its subunits, indicating that they are not strongly associated. The substrates displace the equilibrium toward the dimer, which is the native form. Thiol groups are essential for both the structure and the activity of glycyl-tRNA synthetase. All these results will be discussed in light of their possible physiological significance.

Experimental Procedures

Products. [^{14}C]- and [^{3}H]glycine (200 mCi/mmol and 35 Ci/mmol, respectively) and Na[^{32}P]PP_i were from the Commissariat à l'Energie Atomique. DEAE-cellulose (DE-52) was from Whatman; hydroxylapatite (Bio-Gel HTP) was from Bio-Rad Laboratories; Ultrogel ACA34 was from the Industrie Biologique Française. Bovine serum albumin (Cohn, fraction V) and other protein markers (ovalbumin, alcohol dehydrogenase, catalase, lysozyme), DIFP, PMSF, DTE,[1] and ATP (crystalline disodium salt) were from Sigma. All other products were of the highest purity commerically available.

Yeast and tRNAs. The synthetase was extracted either from cells at the stationary phase by using fresh commercial baker's yeast cakes provided by the Société Industrielle de Levure Fala or from cells at the exponential phase according to Kern et al. (1975).

Unfractionated tRNA from brewer's yeast was from Boehringer. Pure $tRNA^{Gly}$ (major species) was obtained after

† From the Laboratoire de Biochimie, Institut de Biologie Moléculaire et Cellulaire du CNRS, 67084 Strasbourg Cedex, France. *Received January 10, 1980; revised manuscript received August 1, 1980.* This work was supported by grants from the Délégation Générale à la Recherche Scientifique et Technique and from the Centre National de la Recherche Scientifique. Preliminary results of this work were included in the thesis (3ème Cycle) of D. Kern (Strasbourg, 1972).

[1] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Na_2EDTA, disodium (ethylenedinitrilo)tetraacetate; DIFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; DTE, dithioerythritol; DTT, dithiothreitol; GSH, reduced glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

0006-2960/81/0420-0122$01.00/0

countercurrent distribution (Dirheimer & Ebel, 1967), followed by two successive BD-cellulose columns at pH 7.0 and 4.0 (Gillam et al., 1967) and by reverse-phase chromatography (RPC no. 5) according to Pearson et al. (1971).

General Procedures for Handling of Proteins. The synthetase was purified at 4 °C as quickly as possible in order to reduce enzyme denaturation. All buffers used contained 10% glycerol, 0.1 mM NaEDTA,[1] 5 mM β-mercaptoethanol, and DTE, PMSF, and/or DIFP as indicated. Protease inhibitors were prepared as stock solutions (0.1 M) in 2-propanol and were added to the cooled buffers just before use. After each purification step, the active fractions were dialyzed against 20 mM potassium phosphate buffer at pH 7.2.

The amounts of proteins in the enriched or pure synthetase fractions were determined by using the colorimetric method of Lowry et al. (1951). The protein fractions were concentrated under vacuum in Ultra Thimbles (Schleicher & Schüll) to final volumes of 2–10 mL. Pure glycyl-tRNA synthetase was further concentrated by dialysis against buffers containing 70% glycerol. The enzyme were stored at −20 °C.

Molecular Weight Determinations. Sucrose gradient centrifugations were performed under the conditions described previously (Kern et al., 1979). Electrophoresis under nondenaturing conditions (6% acrylamide, pH 6.8, or 7.5% acrylamide, pH 8.9) was conducted as described by Davis (1964) except that 1 mM thioglycholate was present in the gels and that before electrophoresis the gels were dialyzed against 5 mM DTE. Electrophoresis under denaturing conditions (7.5% acrylamide, pH 7.2 or 8.9) were done as described by Shapiro et al. (1967) and Weber & Osborn (1969). Migrations were carried out at 4 or 25 °C under nondenaturing or denaturing conditions, respectively.

Glycyl-tRNA Synthetase Activities. (*A*) *Aminoacylation of tRNA.* Unless otherwise indicated, the incubation mixtures contained 50 mM Na Hepes,[1] pH 7.2, 15 mM $MgCl_2$, 10 mM ATP, 20 mM DTE, 100 mM KCl, 0.1 mM [^{14}C]glycine (25 mCi/mmol), 200 μg of bovine serum albumin/mL, 5.0 mg of unfractionated yeast tRNA/mL, and enzyme. Incubation was carried out at 30 °C. The rates of Gly-tRNA formation were determined as described previously (Kern et al., 1975).

The enzymatic activity unit is the amount of protein which catalyzes the synthesis of 1 nmol of Gly-tRNA/min under the above described conditions. Specific activities are expressed as the number of enzymatic units per milligram of proteins.

(*B*) [^{32}*P*]*PP$_i$–ATP Isotope Exchange.* The incubation mixtures contained 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, 2 mM Na[^{32}P]PP_i (about 1000 cpm/nmol), and 1 mM glycine. In some cases, tRNA from yeast and/or DTE was present at the indicated concentrations. The reactions were conducted at 35 °C, and the rates of [^{32}P]ATP synthesis were determined as described previously (Kern et al., 1979).

(*C*) *Initial Rate Measurements of Glycyl-tRNA Synthetase Activities after Partial Dissociation of the Enzyme.* Because of the exponential kinetics observed for both activities resulting from the reactivation of the synthetase by the substrates (see Results), the initial rates were measured during sufficiently short incubation times to minimize the substrate-induced reactivations. The aminoacylation mixtures contained 100 mM NaHepes, pH 7.2, 100 mM KCl, 5 mM ATP, 7.5 mM $MgCl_2$, 2 mM [^{3}H]glycine (105 cpm/pmol), 50 μM $tRNA^{Gly}$, and the indicated enzyme concentration. After 15–60 s of incubation at the indicated temperature, 100–500-μL aliquots were transferred to 2 mL of a solution of 10% trichloroacetic acid. After filtration on glass filter disks (GF/c Whatman), the [^{3}H]Gly-$tRNA^{Gly}$ synthesized was determined as described previously (Kern et al., 1975). The isotope-exchange mixtures were as described above. The [^{32}P]ATP synthesized was measured in 100–500-μL aliquots after 15–60 s of incubation.

Purification Procedures. The first attempts of purification, conducted in the absence of proteases inhibitors and DTE, were unsuccessful because of the rapid inactivation of the synthetase. Three specific procedures, using these protectors, were therefore developed.

Procedure 1. This procedure started with 2 kg of cells. DTE (0.1 mM) was present throughout the purification. The steps up to DEAE-cellulose column chromatography were those described by Kern et al. (1975). Pure enzyme was obtained after two further sets of column chromatography on CM-Sephadex (4.2 × 42 cm; elution with a linear gradient of 2× 500 mL from 0.1 to 0.3 M KCl containing 0.02 M potassium phosphate, pH 6.5) and hydroxylapatite [2.5 × 35 cm; elution with a linear gradient of 2× 500 mL of potassium phosphate from 0.02 to 0.5 M, pH 6.5 (Figure 1A)].

Procedure 2. This procedure was similar to the first one except that 10 mM PMSF was present during cell disruption (1 mM in the further steps) and the concentration of DTE was increased to 1 mM. After DEAE-cellulose chromatography, glycyl-tRNA synthetase was further purified by column chromatography on phosphocellulose (3 × 28 cm; elution with a linear gradient of 2× 500 mL from 0.1 to 0.3 M KCl containing 0.01 M potassium phosphate, pH 7.0) and hydroxylapatite [1.8 × 9 cm; elution with a linear gradient of 2× 100 mL of potassium phosphate from 0.02 M, pH 8.0, to 0.9 M, pH 6.5 (Figure 1B)].

Procedure 3. In this case, 20 kg of cells was processed. DIFP, PMSF, and DTE were present at 1 mM in the cell-disruption buffer. In the other buffers, the concentration of DIFP was reduced to 0.1 mM whereas those of PMSF and DTE remained unchanged. The steps up to DEAE-cellulose chromatography were as described by Kern et al. (1977). Purification was achieved by column chromatography on hydroxylapatite (10 × 15 cm; elution with a linear gradient of 2× 2 L from 0.02 to 0.8 M potassium phosphate, pH 7.2) and phosphocellulose (7 × 37 cm; elution with a linear gradient of 2× 3 L from 0 to 0.8 M KCl containing 0.01 M potassium phosphate, pH 7.0) followed by gel filtration on Ultrogel ACA34 (Figure 1C). The Ultrogel column (2 × 210 cm) was equilibrated with 50 mM NaHepes, pH 7.2, 1 mM DTE, 0.2 mM DIFP, and 0.1 mM Na_2EDTA without KCl (C) or with 1.0 M KCl (C′) and loaded with half of the proteins of the phosphocellulose fraction (in a volume of about 10 mL previously dialyzed against the equilibrating buffer). The flow rate was 20 mL/h, and 4.0-mL fractions were collected.

Results

Purification of Glycyl-tRNA Synthetase. Table I summarizes the three purification procedures of glycyl-tRNA synthetase. It shows that pure enzymes of increasing specific aminoacylation activities can be obtained in the presence of increasing concentrations of DIFP, PMSF, and DTE all the way through the purification procedure.

After the last purification step, the enzyme was eluted as an apparently homogeneous peak in procedures 1 and 2 (Figure 1A,B). In procedure 3, however, two elution patterns were obtained depending on whether the gel filtration on Ultrogel ACA34 was done in the absence or in the presence of 1 M KCl. In the absence of salt, the synthetase was eluted as a homogeneous peak of constant specific activity (Figure 1C) whereas in the presence of KCl, two peaks appeared, but only one exhibited glycyl-tRNA synthetase activity (Figure 1C, inset).

Table I: Purifications of Glycyl-tRNA Synthetase

step	total protein (mg)	units (× 10^3)	specific activity (units/mg)	purification ratio	yield (%)
Procedure 1 (0.1 mM DTE without protease inhibitors)[a]					
1. crude extract	85 200	14.7	0.17	1.0	100
2. 105000*g*	68 500	13.2	0.19	1.13	90
3. $(NH_4)_2SO_4$	14 800	7.55	0.51	3.0	52
4. DEAE-cellulose	1 020	1.35	1.33	7.8	9.2
5. CM-Sephadex	224	0.81	3.6	21.1	5.5
6. hydroxylapatite	29	0.21	7.4	43.5	1.5
Procedure 2 (1 mM PMSF and 1 mM DTE)[b]					
1. crude extract	80 000	20.0	0.25	1.0	100
2. 105000*g*	63 000	17.9	0.28	1.14	89
3. $(NH_4)_2SO_4$	13 300	14.8	1.11	4.45	74
4. DEAE-cellulose	1 021	8.36	8.18	32.7	42
5. phosphocellulose	221	4.47	20.2	80.9	22
6. hydroxylapatite	19	2.02	106.3	425.2	10
Procedure 3 (0.1 mM DIFP, 1 mM PMSF, and 1 mM DTE)[c]					
1. crude extract	350×10^3	4.6×10^5	0.34	1.0	100
2. $(NH_4)_2SO_4$	244×10^3	3.4×10^5	1.39	4.1	74
3. Sephadex G-200	130×10^3	2.4×10^5	1.88	5.54	53
4. DEAE-cellulose	12×10^3	1.4×10^5	11.86	34.9	30
5. hydroxylapatite	1270	1.0×10^5	81.5	240	22
6. phosphocellulose	330	8.1×10^4	246	723	18
7. Ultrogel ACA34	145	7.9×10^4	550	1618	17

[a] From 2 kg of commercial baker's yeast. [b] From 2 kg of exponential baker's yeast. [c] From 20 kg of exponential baker's yeast.

The purity of the protein fractions after the last step was checked by electrophoresis on polyacylamide gels. Various patterns were observed. Procedure 1 yields a homogeneous enzyme. This is not the case for the two other procedures. For the apparently pure preparation obtained in the presence of PMSF and DTE (procedure 2), two major protein bands and one minor protein band were found; these three bands were reduced to one in the presence of $tRNA^{Gly}$ (Figure 1B). Electrophoresis of the Ultrogel ACA34 fractions (procedure 3) showed different results depending on whether KCl was present. In the presence of KCl, the proteins migrate as an unique band at pH 6.8 and as two bands of equal intensity at pH 8.9 (Figure 1C). In the absence of KCl, electrophoresis of each peak revealed one band.

Purified enzyme preparations were also examined for the presence of possible contaminating aminoacyl-tRNA synthetases. Less than 5% of contaminating activities (alanyl-, isoleucyl-, and valyl-tRNA synthetases) were detected in the glycyl-tRNA synthetases obtained by procedures 1 and 2. These contaminants were approximately 1% for the synthetase obtained according to procedure 3.

Molecular Weight and Oligomeric Structure of Glycyl-tRNA Synthetase. Significant variations in the molecular weight and subunit structure of glycyl-tRNA synthetase were found. Table II shows that the synthetase obtained in the absence of proteases inhibitors (procedure 1) is a tetramer of the $\alpha_2\beta_2$ type (α = 67 600, β = 57 500). In the presence of these protectors, heterogeneous populations of dimeric type were found. Two dimers (α = 140 000 and α'_2 = 112 000), in an approximately 20:1 ratio, were obtained by procedure 2. The polymers, especially those of the α_2 type, are probably partly dissociated into monomers as indicated by the existence of a band of low molecular weight (72 500 daltons) observed under nondenaturing conditions. After purification under optimal protecting conditions (procedure 3), a homogeneous α_2 population of molecular weight 158 000 was observed.

Association Properties of Glycyl-tRNA Synthetase with Other Proteins. This study was done with the synthetase purified according to procedure 3. Filtration of the phosphocellulose fraction of the synthetase on Ultrogel in the absence of KCl resulted in coelution of glycyl- and valyl-tRNA synthetases in a complex of apparent molecular weight ~ 280 000 whereas in the presence of 1.0 M KCl both activities eluted separately with apparent molecular weights of 80 000 and 150 000, respectively (Figure 2). This was confirmed by sucrose gradient centrifugation and by disc gel electrophoresis under nondenaturing conditions. Several lines of experimental evidence, however, seem to indicate that the association between the two synthetases is not specific. For example, the Ultrogel fraction of glycyl-tRNA synthetase obtained in the absence of KCl only contained 10%–20% of contaminating valyl-tRNA synthetase activity excluding a 1:1 stoichiometric association between the two enzymes; consequently, the lower band observed on the gels at pH 8.9 (see Figure 1C) is likely to contain other proteins in addition to valyl-tRNA synthetase. Furthermore, when the less enriched DEAE-cellulose fraction was analyzed on Ultrogel in the absence of KCl, glycyl-tRNA synthetase also eluted with an apparent molecular weight of about 280 000, but, in contrast to the previous experiment, the contaminating valyl-tRNA synthetase activity represented only ~5% of that of the glycine enzyme. Among the other proteins which coeluted with glycyl-tRNA synthetase, we detected alanyl-, leucyl-, methionyl-, and tyrosyl-tRNA synthetases,

Table II: Molecular Weights of Glycyl-tRNA Synthetases Determined by Various Techniques

technique[a]	glycyl-tRNA synthetase purified according to procedure 1	2	3
sucrose-gradient centrifugation	210 000	117 000–136 000	135 000–142 000
electrophoresis (nondenaturing)	250 000	140 000[c] 72 500[c] 120 000[c]	158 000
electrophoresis (denaturing)	α = 67 600[b] β = 57 500[b]	72 000[c] 64 000[c]	80 000
Ultrogel ACA34 gel filtration	250 000	140 000	160 000

[a] The conditions are given under the Experimental Procedures. [b] Two bands of equal intensity were shown. [c] The molecular weights are classified according to decreasing intensity of the protein bands.

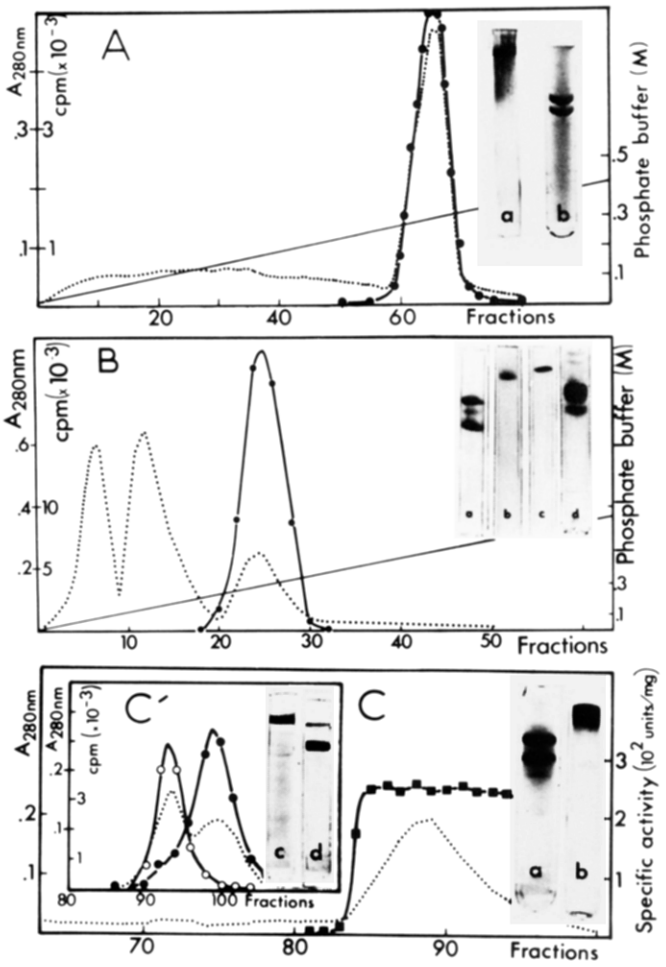

FIGURE 1: The last steps of purification of glycyl-tRNA synthetase. (A, B) Elution of the enzyme on hydroxylapatite according to purification procedures 1 and 2. (C, C') Filtration of 8 mg of proteins of the phosphocellulose fraction on Ultrogel ACA34 in the absence (C) or the presence (C') of 1.0 M KCl. Each fraction was diluted 10 (A), 20 (B), or 200 (C, C') times in the aminoacylation mixture, and the amounts of Gly-tRNA^{Gly} were determined after 10 min of incubation at 30 °C. Valyl-tRNA synthetase was tested as described by Kern et al. (1975). When the filtration of glycyl-tRNA synthetase was performed in the presence of 1.0 M KCl (C'), the enzyme was tested after a 10-fold dilution of each fraction with a buffer devoid of KCl but containing 1 μM tRNA^{Gly} (in unfractionated yeast tRNA) and a preincubation of 10 min at 30 °C. (···) A_{280nm}; (—) molarity of phosphate buffer; (●) glycyl- or (○) valyl-tRNA synthetase activities; (■) specific activity of glycyl-tRNA synthetase. The inset figures show polyacrylamide gels of the various glycyl-tRNA synthetase fractions (20–50 μg of proteins). (A) Under nondenaturing (a) or denaturing (b) conditions (7.5% gels, pH 8.9, 0.4 M Tris–HCl). (B) Under nondenaturing (a–c) or denaturing (d) conditions: (a) 7.5% gels, pH 8.9 (0.4 M Tris-HCl); (b,c) 6% gels, pH 6.8 (5 mM NaHepes, 10 mM NH_4Cl, 5 mM magnesium acetate); (c) 20 μg of tRNA^{gly} was added; (d) 7.5% gels, pH 7.2. (C) Under nondenaturing conditions: (a) 7.5% gels, pH 8.9 (0.4 M Tris-HCl); (b) 6% gels, pH 6.8 (5 mM NaHepes). (C') Under nondenaturing conditions (7.5% gels, pH 8.9, 0.4 M Tris-HCl): (c) glycyl-tRNA synthetase rich fraction; (d) valyl-tRNA synthetase rich fraction. Before analysis, fractions c and d were exhaustively dialyzed against the Ultrogel-equilibration buffer deprived of KCl and were concentrated.

but their activity did not exceed a few percent of their total activity.

Optimal Aminoacylation Conditions and Kinetic Constants. The rate of aminoacylation of tRNA^{Gly} was optimal in a temperature range from 35 to 40 °C at pH 7.2 (in NaHepes or sodium cacodylate buffer) in the presence of 100 mM KCl and a $MgCl_2$/ATP ratio of 1.5. Other bivalent cations or spermidine did not efficiently replace $MgCl_2$. Protectors of

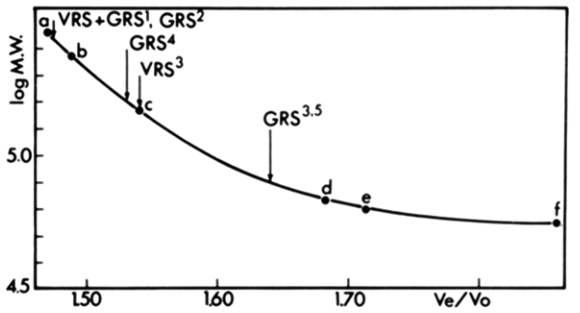

FIGURE 2: Determination of the molecular weight of glycyl-tRNA synthetase on Ultrogel ACA34. The filtration was conducted as in Figure 1. The curve represents the variation of V_e/V_0 as a function of the log of molecular weight; V_0 (241 mL) corresponds to the elution volume of dextran blue and V_e corresponds to that of the vaiours proteins: (a) yeast phenylalanyl-tRNA synthetase, (b) catalase, (c) alcohol dehydrogenase, (d) bovine serum albumin, (e) hemoglobin, (f) yeast arginyl-tRNA synthetase; (1) phosphocellulose fraction of glycyl-tRNA synthetase in the absence of KCl, (2) DEAE-cellulose fraction in the absence of KCl, (3) phosphocellulose fraction in the presence of 1.0 M KCl, (4) purified synthetase (after gel filtration in the presence of KCl) in the absence of KCl, (5) purified synthetase in the absence of KCl and in the absence of DTE. In the last experiment before filtration, glycyl-tRNA synthetase was preincubated for 5 h at 35 °C in the presence of 50 mM NaHepes, pH 7.2, 0.2 mM DIFP, and 0.1 mM Na_2EDTA. GRS and VRS represent, respectively, glycyl- and valyl-tRNA synthetases.

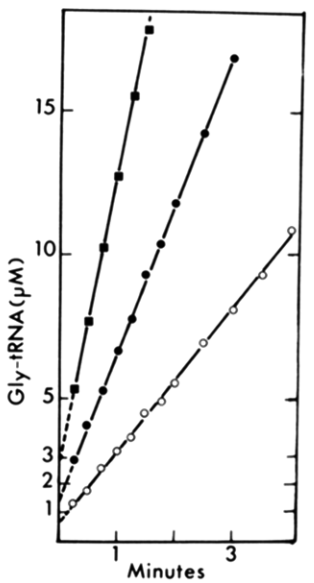

FIGURE 3: Kinetics of charging of tRNA^{Gly} in the presence of high enzyme concentrations. The incubation mixtures contained 100 mM NaMes[1], pH 6.2, 5 mM ATP, 7.5 mM $MgCl_2$, 2.0 mM [^{3}H]glycine (55 400 cpm/nmol), 100 μM tRNA^{Gly}, and 1.4 (■), 0.7 (●), or 0.35 μM (○) glycyl-tRNA synthetase. The reactions were conducted at 4 °C, and the amounts of labeled aminoacyl-tRNA were synthesized after various incubation times determined in 50-μL aliquots as described under the Experimental Procedures.

SH groups (DTE, DTT, or GSH)[1] at a concentration of 20 mM were required to obtain an optimal aminoacylation rate (see following section).

The apparent K_m values of the synthetase for tRNA^{Gly}, ATP, and glycine determined at pH 7.2 are 8.8×10^{-8}, 2.1×10^{-5}, and 2.5×10^{-4} M, respectively. At pH 7.2 and in the presence of saturating substrate concentrations, a turnover number of 6.0 s^{-1} was measured. The rate-determining step of tRNA aminoacylation was investigated by analysis of the kinetics performed in the presence of saturating substrate concentrations under conditions where the first catalytic cycles of the enzyme could be followed. Biphasic kinetics were ob-

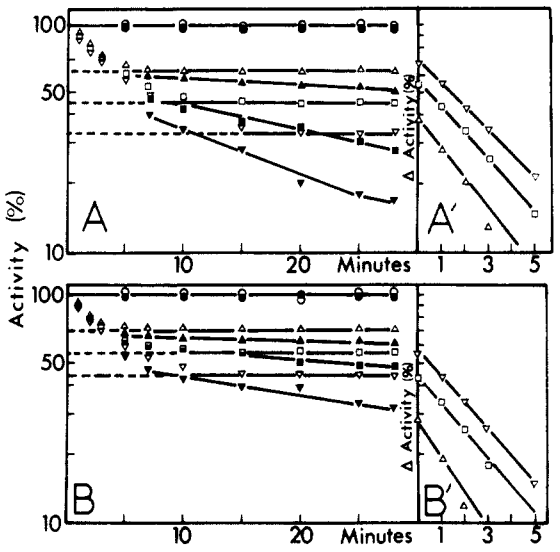

FIGURE 4: Kinetics of inactivation of glycyl-tRNA synthetase at high dilutions. The enzyme (stock solution: 1.56 μM in 100 mM NaHepes, pH 7.2, 0.2 mM DIFP, 0.1 mM Na_2EDTA, and 0.1 mM DTE) was preincubated at 25 °C without dilution before (○) or after adjustment (●) of the concentration of DTE to 20 mM, or after dilution in the same buffer devoid (▲, ■, ▼) or containing 20 mM DTE (△, □, ▽). At various time invervals, aliquots were removed and tested for the remaining enzyme activities. (A, A′) tRNA charging activity. The concentrations of synthetase in the preincubation mixtures were 1560 (○, ●), 15.6 (△, ▲), 5.2 (□, ■), and 2.6 nM (▽, ▼). After various preincubation times, the initial rates were determined at 25 °C as described under Experimental Procedures after an additional 100- (○, ●) or 2-fold (△, ▲, □, ■, ▽, ▼) dilution of the enzyme in a complete aminoacylation mixture. (B, B′) [^{32}P]PP_i–ATP exchange activity. The concentrations of the enzyme during the preincubation were 1560 (○, ●), 31.2 (△, ▲), 10.4 (□, ■), and 5.2 nM (▽, ▼). After various preincubation times, the initial rates were determined at 25 °C as described under Experimental Procedures. (A) and (B) represent the dependence of the remaining tRNA charging and isotope-exchange activities of the synthetase upon the preincubation time. (A′) and (B′) are first-order plots of the relative time-dependent enzyme inactivation shown in (A) and (B) in the presence of DTE (i.e., the difference between the experimental values of the remaining activities and the extrapolation to zero time of the linear phase).

tained (Figure 3). The Gly-tRNA^{Gly} synthesized during the first (fast) phase was in a 2:1 stoichiometric ratio with the synthetase present.

Effects of Protease Inhibitors and DTE on the Stability of the Synthetase. Table I clearly shows that glycyl-tRNA synthetase can be purified with high yield and specific activity only in the presence of protease inhibitors and DTE. A systematic study of the effects of protectors of thiol groups (DTE, DTT and GSH) on the aminoacylation and the [^{32}P]PP_i–ATP isotope-exchange reactions showed inactivation kinetics in the absence of these protectors and reactivation kinetics in their presence at a 20 mM concentration. Strikingly, β-mercaptoethanol did not induce any stimulation in the concentration range 0.1–100 mM. As shown in Table III, the extent of reactivation of the synthetase by DTE is dependent on the presence of protease inhibitors during purification and storage of the enzyme as well as on the age of the enzyme solution. Three conclusions concerning the reactivation of the synthetase can be drawn, whatever its extent of purification: (i) the presence of protease inhibitors during purification is not sufficient to protect the enzyme against inactivation but is necessary to obtain its reactivation by DTE. The reactivation occurs either by preincubation of the synthetase for ~10 min at 20 °C in the presence of 20 mM DTE (after which linear kinetics are obtained) or by addition of the protector in the aminoacylation or in the isotope-exchange mixtures (in which case exponential kinetics are observed). (ii) The absence of

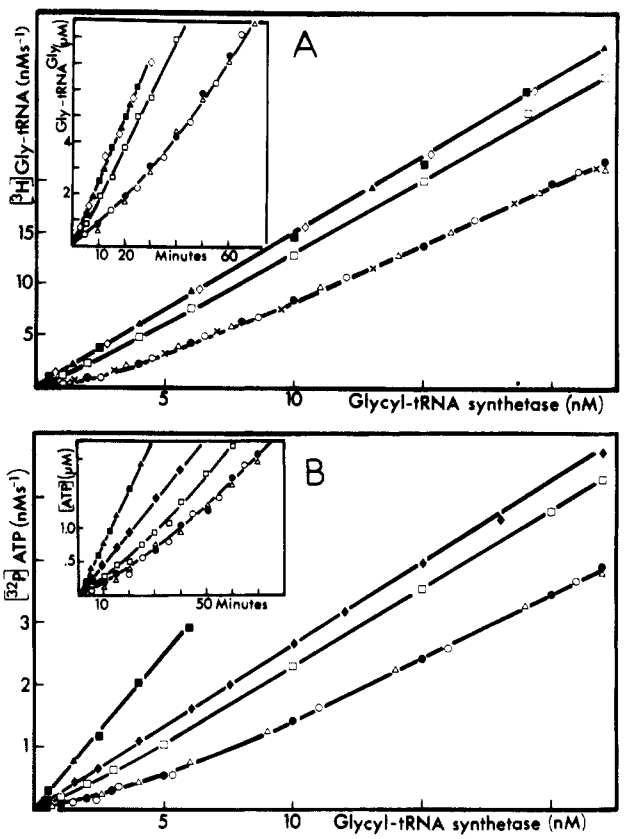

FIGURE 5: Evolution of the glycyl-tRNA synthetase activities as function upon the enzyme concentration in the preincubation mixture and effect of substrates. (A) tRNA charging. (B) PP_i–ATP exchange. The stock solution of enzyme (1.56 μM in the buffer described in Figure 4) was diluted in the same buffer containing 20 mM DTE either in the absence of substrates (△) or in the presence of 5 mM ATP (○), 2 mM glycine (●), 0.1 mM Na_2EDTA and 5 mM ATP plus 2 mM glycine (□), 5 mM ATP·$MgCl_2$ plus 2 mM glycine (◇), 1 μM tRNA^{Gly} (▲), or 1 μM tRNA^{Phe} (X). The glycine was either ^{3}H labeled (with the same specific activity as in the aminoacylation mixture) when the tRNA charging was tested or not labeled when the isotope exchange was tested. After 20 min of preincubation at 25 °C, the initial rates of both activities were determined at 25 °C, as described under the Experimental Procedures, in reaction mixtures where the enzyme was further diluted twice. In control experiments, the enzyme was directly diluted in the aminoacylation (■) or in the isotope-exchange mixture containing (■) or not containing (◆) 1 μM tRNA^{Gly}. The inset figures represent, respectively, the kinetics of aminoacylation (A) and of isotope exchange (B) after dilution of the synthetase to 2.6 nm and preincubation for 20 min at 25 °C under the conditions described above.

protease inhibitors during purification results in an irreversible inactivation of the synthetase, even when DTE is present. (iii) Both tRNA charging and isotope-exchange activities are lost in the absence of protease inhibitors, but the tRNA-charging activity decreases more rapidly in the absence of DTE than does the isotope-exchange activity.

Inactivation by Dilution and Reactivation of the Catalytic Activities of Glycyl-tRNA Synthetase. The enzyme was diluted at 25 °C to a low concentration (in the range 0.5–30 nM), and its tRNA charging activity was tested by initial rate measurements as a function of preincubation times; biphasic inactivation kinetics were observed (Figure 4A). The relative amplitude of the first rapid phase was varied inversely with the enzyme concentration, but the time necessary for completion of this phase was independent of this concentration. Similar inactivation kinetics were observed for the [^{32}P]-PP_i–ATP exchange reaction (Figure 4B). The inactivation of both activities during the fast phase was first order (Figure 4). For the same dilution of the enzyme, the amplitudes of

Table III: Reactivation of Glycyl-tRNA Synthetase Activities after Partial or Complete Purification under Various Conditions

enzyme fraction	specific activity[a] (units/mg)					
	aminoacylation			PP_i-ATP isotope exchange		
	before reactivation[b]	after reactivation[c]	extent of reactivation	before reactivation[b]	after reactivation[c]	extent of reactivation
DEAE-cellulose[d,h]	0.80	0.96	1.2	0.54	0.55	1.0
DEAE-cellulose[d,i]	0.01	0.01	1.0	0.01	0.01	1.0
DEAE-cellulose[e,h]	4.12	11.13	2.7	4.20	6.70	1.6
DEAE-cellulose[e,i]	0.10	10.50	105.0	0.23	6.70	29.1
pure[f,h]	7.40	10.40	1.4	6.05	6.20	1.0
pure[f,i]	0.06	0.07	1.1	0.04	0.04	1.0
pure[g,h]	550.0	580.0	1.0	330.0	330.0	1.0
pure[g,i]	570.0	580.0	1.0	330.0	330.0	1.0

[a] Specific activities were determined from initial rate measurements in a complete aminoacylation or [^{32}P]PP_i-ATP isotope-exchange mixture, not containing[b] or containing[c] 10 mM DTE. [c] Reactivations were carried out by preincubating the synthetase during 10 min at 35 °C in the presence of 20 mM DTE. [d] DEAE-cellulose fraction obtained in the absence of protease inhibitors and DTE. [e] DEAE-cellulose fraction obtained in the presence of 1 mM DIFP and PMSF but in the absence of DTE. [f] Synthetase purified according to procedure 1. [g] Synthetase purified according to procedure 3. The measurements are performed either immediately[h] or after 3-months' storage at −20 °C in the presence of protease inhibitors and DTE at the concentrations present along the purification[i].

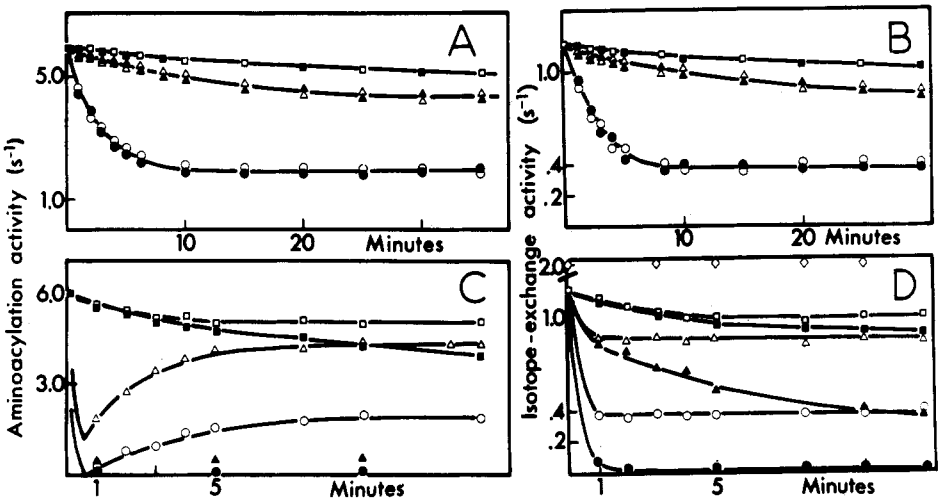

FIGURE 6: Effect of KCl on the tRNA charging and the PP_i–ATP isotope-exchange activities of glycyl-tRNA synthetase in the absence and in the presence of DTE. The stock solution of enzyme (1.56 μM in the buffer described in Figure 4) was diluted to 93.6 nM in the same buffer containing (□, △, ○, ◇) or not (■, ▲, ●) 20 mM DTE in the absence of KCl (□, ■) or in the presence of 0.1 M KCl (△, ▲) or 1 M KCl (○, ●). In one experiment (◇), 1 μM $tRNA^{Gly}$ was present in the preincubation mixture. After various preincubation times either at 4 °C (A, B) or at 35 °C (C, D), aliquots were transferred into a complete reaction mixture at 35 °C where the enzyme was further diluted 10 times; the final KCl concentrations were 0.1 M. The initial rates of aminoacylation (A, C) and of isotope exchange (B, D) were determined as described under Experimental Procedures.

decrease in both tRNA charging and isotope-exchange activities during the fast phase of inactivation were similar. However, isotope exchange decreased slower during the second (slow) phase than the tRNA charging. The second phase of inactivation of both activities was entirely suppressed when DTE or GSH was present in the preincubation mixture (Figure 4).

The presence of glycine or ATP alone during preincubation did not affect the dilution-promoted inactivation of the synthetase (Figure 5). However, the simultaneous presence of both substrates significantly reduced the extent of inactivation of the two activities. This was also the case for ATP·$MgCl_2$ plus glycine or $tRNA^{Gly}$. Indeed, when enzyme was preincubated in the presence of these substrates, a linear relationship between both activities and enzyme concentration was observed. $tRNA^{Phe}$ could not replace $tRNA^{Gly}$ in this protection (Figure 5). ATP·$MgCl_2$ plus glycine and $tRNA^{Gly}$ reversed the inactivation of the enzyme (inserts in Figure 5). The lag period, which corresponds to the exponential increase of the tRNA charging and isotope-exchange rates, decreased when the enzyme concentration was increased in the preincubation mixture. This lag period was not affected when either ATP or glycine was present during preincubation; however, the presence of both substrates reduced it significantly. Linear tRNA charging and isotope-exchange kinetics were obtained whether the synthetase was preincubated at a low concentration in the presence of ATP·$MgCl_2$ plus glycine or $tRNA^{Gly}$ or directly diluted into the reaction mixture (inserts in Figure 5). In addition to its protecting effect against the dilution-promoted inactivation, $tRNA^{Gly}$ stimulated isotope exchange about 2-fold regardless of the enzyme concentration. This stimulation was not a consequence of a tRNA effect on the magnesium concentration since in the range 1–17 mM $MgCl_2$, $tRNA^{Gly}$ stimulated twice the isotope exchange whereas $tRNA^{Phe}$ inhibited it (results not shown). Finally, the substrate-induced reactivation of glycyl-tRNA synthetase following its dilution occurred only when DTE or GSH was present during preincubation (results not shown).

Existence of an Equilibrium between Active Dimers and Inactive Monomers of Glycyl-tRNA Synthetase. The previous results can be interpreted in the light of the existence of an equilibrium between two forms of glycyl-tRNA synthetase; the first one capable of catalyzing tRNA charging and isotope exchange and a second inactive one (see the Appendix). ATP and glycine individually do not affect this equilibrium whereas both substrates together (in the absence or presence of $MgCl_2$)

as well as $tRNA^{Gly}$ displace it toward the dimers. The reversion from the inactive monomers toward the active dimers only occurs when the synthetase is protected by DTE or GSH. The existence of a lag in the kinetics of aminoacylation and isotope exchange following high dilution of the enzyme in the absence of substrates indicates that the rate of reassociation of the monomers is slow compared with either catalytic activities.

Effect of Salts and of Thiol Protectors on the Equilibrium between Monomers and Dimers. Figure 6 shows that KCl increases the rate and the extent of inactivation of the tRNA charging and isotope-exchange activities of the diluted enzyme. This salt effect is reversible (not shown). At 0 °C, it was not affected by DTE (Figure 6A,B). However, at 35 °C, the kinetics of inactivation in the presence of salt were different depending on whether or not the preincubation mixture contained DTE and GSH (Figure 6C,D). In the absence of these protectors, preincubation of the synthetase in the presence of KCl resulted in a strong and fast decrease of its aminoacylation activity; during the first minute of preincubation, 90% and 100% of this activity were lost (only 25% and 70% at 0 °C) in the presence of 0.1 and 1.0 M KCl, respectively (Figure 6C). When SH group protectors were present in the preincubation mixture, the inactivation of the aminoacylation activity was only 25% and 70% in the presence of 0.1 and 1.0 M KCl, respectively. However, as shown in Figure 6C, the kinetics were particular: a strong and fast decrease in the aminoacylation activity occurred during the first minute of preincubation, followed by a slow and partial recovery of this activity. When the isotope-exchange capacity was tested after preincubation of the enzyme under similar conditions, biphasic kinetics were observed. The rates of the first (fast) phase and the second (slow) phase of inactivation increased with increasing KCl concentrations (Figure 6D). The presence of 20 mM DTE significantly decreased the rate of inactivation of the second (slow) phase whatever the concentration of KCl was present. Finally, the optimal extent of the KCl-induced inactivation of the two activities was the same whether the synthetase was incubated at 0 °C in the absence or in the presence of SH group protectors or at 35 °C in their presence (Figure 6A–D). NaCl and NH_4Cl exhibited similar effects (results not shown).

Titration of the Thiol Groups of Glycyl-tRNA Synthetase. In the native enzyme, four SH residues were titrated by DTNB[1] in less than 15 s; this resulted in a complete loss of the tRNA charging activity. Other SH groups were slowly titrated, and this was accompanied by a parallel decrease in the isotope-exchange activity. In the presence of 1 M KCl or 6 M guanidine hydrochloride, 8 and 18 SH residues, respectively, reacted rapidly with DTNB, also leading to inactivation of the synthetase (see Figure 7).

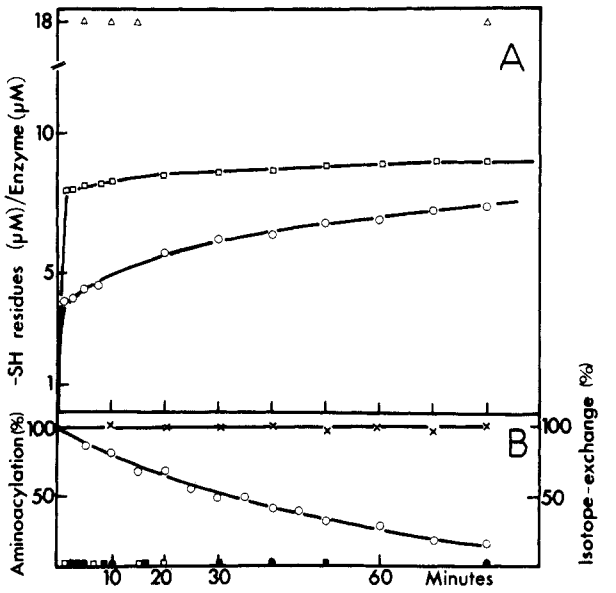

FIGURE 7: Titration of the SH residues of glycyl-tRNA synthetase by DTNB (A) and the effects on the tRNA charging and the isotope-exchange activities (B). Glycyl-tRNA synthetase (156 μM) was dialyzed for 12 h at 4 °C against 100 mM Tris-HCl, pH 7.5, containing a minimal concentration of (10 μM) DTE under a constant nitrogen flow. The enzyme was then diluted 33 times (final concentration 5.2 μM) in 100 mM Tris-HCl, pH 7.5, and 500 μM DTNB either in the absence (○) or in the presence of 6 M guanidine hydrochloride (△) or 1.0 M KCl (□). The A_{412nm} was followed at 25 °C, and the concentration of SH groups having reacted was determined by using an ϵ value for the modified groups of 13 600. The extent of reaction of DTNB with the remaining DTE (0.3 μM) was determined in a control experiment where an aliquot of the dialyzing buffer was treated similarly to the assay. These A_{412nm} values were substracted from those of the assay. Part A represents the number of SH groups per dimeric enzyme having reacted with DTNB as a function of reaction time. Part B shows the remaining enzyme activities tested in parallel experiments conducted under similar conditions. Ten-microliter samples of the enzyme solution (5.2 μM) preincubated at 25 °C in the presence of 500 μM DTNB either in the absence (X, ○, ●) or in the presence of 1.0 M KCl (□, ■) were diluted after various times in 1 mL of 100 mM Tris-HCl, pH 7.5, containing 1 μM $tRNA^{Gly}$ (to prevent further dissociation and to reassociate the nondenatured subunits) at 0 °C. The dilutions were immediately assayed for aminoacylation (●, ■) and isotope-exchange (○, □) activities in standard mixtures. In a control experiment (X), the enzyme was preincubated in the absence of DTNB and KCl and treated as the assays.

Discussion

Up until now, no purification procedure has been reported for yeast glycyl-tRNA synthetase, probably because of the low stability of this enzyme in the absence of protease inhibitors and DTE. The use of protease inhibitors during the course of purification has allowed us to obtain a synthetase of high specific activity which is of the α_2 type (α = 80 000 daltons); most probably it is the native enzyme. However, when the purification is conducted in the absence of protectors, the subunits of the enzyme become smaller, and the oligomeric state of the protein may be either of the α'_2 or of the $\alpha_2\beta_2$ type. The observed variations in the structural properties of glycyl-tRNA synthetase result from proteolytic actions on the native protomers, which in turn modifies their association and catalytic properties. When proteolysis occurs, native protomers are converted to four types of fragments of molecular weight 57 500–72 000 (see Table II). Under nonoptimal conditions of protection against proteolysis, dimeric enzymes are obtained with subunits of either 72 000 or 64 000 daltons. When proteolysis is not prevented, the degradation pattern is even more complex: two types of protomers of molecular weight 67 600 and 57 500 are found which lead to a tetrameric structure of the $\alpha_2\beta_2$ type. This last structure possesses the lowest enzymatic activity; however, it resembles the structures described for the *E. coli* (Ostrem & Berg, 1974) and the *B. brevis* (Surguchov & Surguchova, 1975) enzymes, except that in these two proteins the disymmetry between the classes of subunits is significantly more marked. Since the native and most active enzyme from yeast has a α_2 structure, it can be questioned whether the subunits of tetrameric forms derive from the native protomers or whether one subunit derives from a foreign protein. This possibility cannot be excluded since we have shown that native glycyl-tRNA synthetase can associate, albeit weakly, with other proteins, including valyl-tRNA synthetase and other synthetases. This association may be related to the existence of high molecular weight complexes

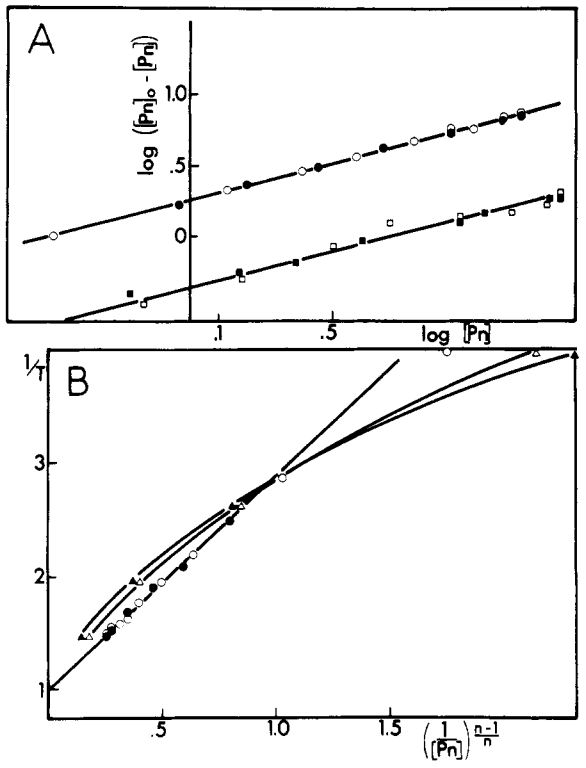

FIGURE 8: Equilibrium of dissociation of glycyl-tRNA synthetase: determinations of n and K_D. The values of $[P_n]_0$ and $[P_n]$ correspond to the experimental values of tRNA charging (○, □) or isotope exchange (●, ■) reported in Figure 5A,B and were analyzed according to eq 1 and 2. (A) Analysis according to eq 1 of the equilibria obtained after dilution of the enzyme in the absence of substrates (○, ●) or in the presence of Na_2EDTA and ATP plus glycine (□, ■). (B) Analysis according to eq 2 by imposing $n = 2$ (○, ●), $n = 3$ (△), or $n = 4$ (▲). In order to clarify the graph, only a few experimental points determined in aminoacylation have been treated and analyzed for $n = 3$ and $n = 4$.

including several synthetases and other components involved in protein synthesis (e.g., Vennegor et al., 1972; Som & Hardesty, 1975; Denney, 1977; Hele & Hebert, 1977; Kellermann et al., 1979).

The monomers of glycyl-tRNA synthetase are catalytically inactive, but their dimerization generates two active sites. This conclusion was derived from a kinetic analysis of tRNA aminoacylation which showed that the first catalytic cycle of tRNA charging is correlated with the synthesis of two Gly-tRNA^{Gly} molecules per dimer. Such a catalytic scheme could agree with the simultaneous binding to the enzyme of two tRNA^{Gly} molecules in a dimeric form as suggested by Wright et al. (1979) after interpretation of the three-dimensional structure of yeast tRNA^{Gly}. This kinetic analysis also enabled us to determine the rate-limiting step of tRNA aminoacylation; as in other systems (Eldred & Schimmel, 1972; Fersht et al., 1978; Kern et al., 1980), this step is the dissociation of the charged tRNA from the protein.

Compared to some other synthetases with oligomeric structures, the two subunits of native glycyl-tRNA synthetase are tightly associated. The dissociation constant (12 nM) is of the same order as that reported for the dimeric tryptophanyl-tRNA synthetase from beef pancreas (Iborra et al., 1973) and methionyl-tRNA synthetase from *E. coli* (Cassio & Waller, 1968) and wheat germ (Chazal et al., 1977). The strong displacement of the equilibrium toward the dimeric form in the presence of substrates indicates that both ATP·$MgCl_2$ plus glycine (when glycyladenylate is formed) and tRNA^{Gly} considerably decrease the dissociation rate of the dimers, probably by strengthening the interaction between the subunits. These interactions are less enhanced by ATP plus glycine when $MgCl_2$ is absent and the adenylate not formed.

In yeast cells, glycyl-tRNA synthetase is present at a concentration of ~0.5 μM. This is significantly higher than the K_D (12 nM) determined under standard in vitro conditions (pH 7.2, in the presence of low ionic strength). On the other hand, the average salt concentration in a crude extract is ~0.2 M (F. Lacroute, personal communication), but this does not preclude significant differences of local conditions as discussed by Douzou & Maurel (1977). Therefore, it may well be that under physiological conditions the K_D of glycyl-tRNA synthetase is in the range of its concentration, thus easily allowing the shift from the inactive toward the active form and vice versa. In this way, conditions would exist that allow a regulation of the synthetase activities by the substrates. Low intracellular concentrations of ATP and/or glycine (when the synthesis of adenylate is not favored) and of tRNA^{Gly} would then displace the equilibrium toward the inactive monomers whereas high substrates concentrations would favor the active dimers. Furthermore, minor variations of enzyme concentration and ionic strength or pH inside the cells could also contribute to this equilibrium.

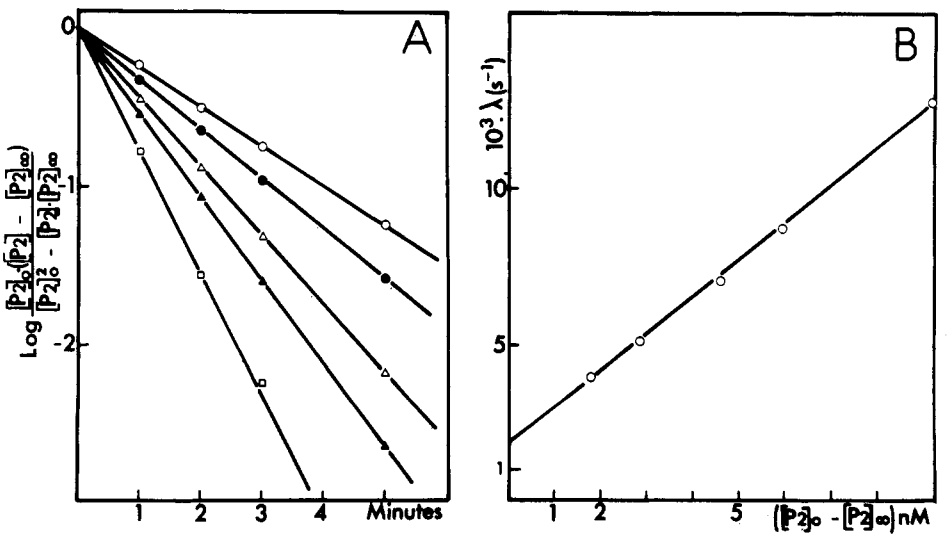

FIGURE 9: Equilibrium of dissociation of glycyl-tRNA synthetase: determinations of k_1 and k_2. The kinetics of dissociation of the synthetase represented in Figure 4A,B were analyzed. The $[P_2]_0$ values were (○) 2.6, (●) 5.2, (△) 10.4, (▲) 15.6, and (□) 31.2 nM. In (A), the data were treated according to eq 4 in order to determine the values of λ. Part B represents the variation of λ as a function of $([P_2]_0 - [P_2]_\infty)$.

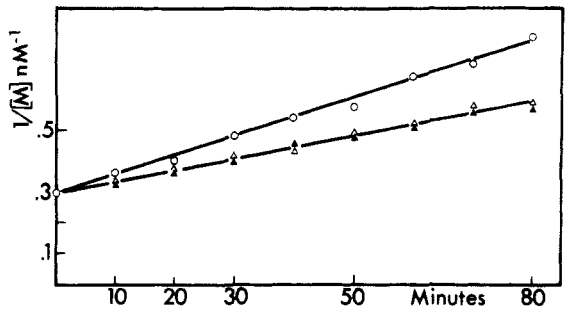

FIGURE 10: Determination of the rates of association of monomers of glycyl-tRNA synthetase in the presence of substrates. The enzyme was diluted to a final concentration of 2.6 nM in the buffer described in Figure 5 containing 20 mM DTE. After 30 min of preincubation at 25 °C, either 2 mM ATP·$MgCl_2$ plus 1 mM glycine (○) or 1 μM $tRNA^{Gly}$ (△, ▲) was added to the preincubation mixture in a negligible volume. At various times, aliquots were transferred into a complete aminoacylation (○, △) or isotope-exchange (▲) mixture, and the initial rates were determined at 25 °C as described under Experimental Procedures. The concentrations of active dimers present after various preincubation times were estimated from the initial rates; the concentration of monomers [M] at a given time t was determined by using the following relation: $[M] = 2([P_2]_0 - [P_2]_t)$ where $[P_2]_0$ and $[P_2]_t$ correspond, respectively, to the analytical enzyme concentration and to the analytical concentration of active dimers. The curve represents the reciprocal of the concentration of monomers as a function of preincubation time.

As reported for most of the aminoacyl-tRNA synthetases (Kisselev & Favorova, 1974), glycyl-tRNA synthetase contains SH groups which are essential for its catalytic activities. However, since only the dimeric form of the synthetase is active and since SH groups are involved in the association between the subunits (because, first, the dimeric synthetase was totally dissociated into monomers unable to reassociate in the absence of DTE or GSH, and, second, the substrates could not generate the dimers in the absence of these protectors), it cannot be concluded whether these essential SH group are involved in the catalytic activities or in the association between the subunits. Nevertheless, we have shown that tRNA charging is more readily affected by oxidative agents of SH groups and DTNB than is isotope exchange. This indicates that different SH groups are involved in the two catalytic activities and suggests that SH groups are directly involved in tRNA charging. KCl increases the accessibility of cysteine residues to oxidative agents by dissociating the dimeric synthetase and by inducing conformational changes of the monomers, both resulting in modifications of the surroundings of these residues. Finally, the high sensitivity of the SH groups to oxidative agents might be of physiological significance. Indeed, glycine participates in the synthesis of GSH, an essential physiological reductor. One can assume that the decrease in the physiological pool of GSH results in an inactivation of glycyl-tRNA synthetase, thus favoring the participation of glycine in the synthesis of GSH.

Acknowledgments

We thank M. Delfau for excellent technical collaboration and Drs. Y. Boulanger and K. Richards for reviewing the manuscript.

Appendix

Establishment of the Equilibrium between Active Dimers and Inactive Monomers of Glycyl-tRNA Synthetase. This study follows the general lines of an eariler analysis by Chazal et al. (1977) on the dissociation into subunits of the methionyl-tRNA synthetase of wheat germ. Various hypotheses have been proposed which were examined for the dilution-induced inactivation of yeast glycyl-tRNA synthetase.

First Hypothesis: Adsorption of the Enzyme on the Glass Tube. This possibility was investigated by using the isotherm equation of Langmuir, which assumes the existence of a linear relationship between the concentration of the active enzyme and its dilution. Parts A and B of figure 6 give values of the remaining synthetase activity as a function of the enzyme dilution. No linear relationship was observed, thus invalidating this hypothesis.

Second Hypothesis: Structural Modification of the Enzyme. (A) Isomerization. This possibility where an active form would be in equilibrium with an inactive one is excluded because such a putative reversible monomolecular reaction would imply a linear proportionality between the rate of reaction (aminoacylation or isotope exchange) of the synthetase and the enzyme concentration when diluted in the absence of substrates. Such proportionality was not observed (Figure 6A,B).

(B) Dissociation. The equilibrium between the oligomers (P_n) and the monomers (P), $P_n \rightleftharpoons nP$, is characterized by a dissociation constant $K_D = [P]^n/[P_n]$. The equation of K_D can be modified as follows:

$$\log ([P_n]_0 - [P_n]) = \log \frac{\sqrt[n]{K_D}}{n} + \frac{1}{n} \log [P_n] \qquad (1)$$

where $[P_n]_0$ and $[P_n]$ correspond, respectively, to the analytical and the effective concentrations of oligomers and n to the number of identical subunits in the oligomers. On the assumption that the monomers are totally inactive, the initial rates of tRNA charging and isotope exchange reported in Figure 5 give values of active dimers as a function of enzyme concentration in the preincubation mixture. The graphical representation of $\log ([P_n]_0 - [P_n])$ as a function of $\log [P_n]$ gives a straight line (Figure 8A), supporting the previous statements. In addition, K_D and n can be determined. Values of 12.6 nM and 2 were found in the absence of substrates. In the presence of ATP plus glycine, K_D decreases to 0.76 nM.

K_D and n can also be determined by considering the fraction T of the active dimeric synthetase, $T = [P_n]/[P_n]_0$, which can be expressed as

$$\frac{1}{T} = \frac{\sqrt[n]{K_D}}{n[P_n]^{(n-1)/n}} \qquad (2)$$

The graphical representation of $1/T$ vs. $1/[P_n]^{(n-1)/n}$ will be linear only for the value of n corresponding to the number of subunits in the oligomer. In our case, this is verified for $n = 2$ (Figure 8B). This approach also gives a K_D value of 13.8 nM.

The rate constants k_1 and k_2 of dissociation and reassociation between monomers and dimers of glycyl-tRNA synthetase can be determined from the differential equation

$$\frac{d[P_2]}{dt} = k_2[P]^2 - k_1[P_2] \qquad (3)$$

where [P] and $[P_2]$ are, respectively, the concentrations of monomers and dimers. After integration, eq 3 becomes

$$\log \frac{[P_2]_0([P_2] - [P_2]_\infty)}{[P_2]^2 - [P_2][P_2]_\infty} = -\lambda t \qquad (4)$$

with

$$\lambda = k_1 + 8k_2([P_2]_0 - [P_2]_\infty)$$

$[P_2]_0$ and $[P_2]_\infty$ being, respectively, the initial concentration

of dimers (at $t = 0$) and the final concentration of dimers after completion of inactivation (at $t \geq 20$ min). Experimental values of dissociation of the synthetase as a function of preincubation time were extracted from the inactivation kinetics presented in Figure 4. Their graphical representation according to eq 4 gives a set of straight lines of slopes λ (Figure 9A). The replot of λ as a function of $([P_2]_0 - [P_2]_\infty)$ gives a straight line (Figure 9B), allowing us to determine k_1 and k_2 values of 1.80×10^{-3} s^{-1} and 0.147×10^6 M^{-1} s^{-1}, respectively. The corresponding K_D value (k_1/k_2) of 12.2 nM is in good agreement with the previously determined values.

The equilibrium between monomers and dimers was found to be dependent on the KCl concentration (Figure 6) and on the pH (not shown). K_D values of 42 nM (at pH 7.2, in the presence of 0.1 M KCl), 550 nM (at pH 7.2, in the presence of 1.0 M KCl), 7.2 nM (at pH 6.5, in the absence of KCl), and 32 nM (at pH 8.5, in the absence of KCl) were calculated.

Finally, the effect of substrates on the association rate constant k_2 has been studied. As the substrates ATP·$MgCl_2$ plus glycine and $tRNA^{Gly}$ completely displace the equilibrium of the synthetase toward its dimers, it can be assumed that the dimers do not dissociate when the substrates are bound. Consequently, association of monomers will be a second-order reaction. The two interacting species being the same, the equation of this reaction will be

$$\frac{1}{[P]} - \frac{1}{[P]_0} = kt \qquad (5)$$

where [P] and $[P]_0$ are, respectively, the concentrations of the monomers at the time t and at the beginning of the reaction (at $t = 0$), and k is the assocation rate constant. The experiments of reassociation of the subunits in the presence of substrates are presented in Figure 10. The slopes (k) of the straight lines allow us to determine a rate constant of 0.1×10^6 M^{-1} s^{-1} in the presence of ATP·$MgCl_2$ plus glycine and of 0.6×10^5 M^{-1} s^{-1} in the presence of $tRNA^{Gly}$ for the association of the subunits.

References

Cassio, D., & Waller, J. P. (1968) *Eur. J. Biochem. 5*, 33–41.

Chazal, P., Thomes, J. C., & Julien, R. (1977) *Eur. J. Biochem. 73*, 607–615.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Denney, R. M. (1977) *Arch. Biochem. Biophys. 183*, 156–167.

Dirheimer, G., & Ebel, J. P. (1967) *Bull. Soc. Chim. Biol. 49*, 1679–1687.

Douzou, P., & Maurel, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1013–1015.

Eldred, E. W., & Schimmel, P. R. (1972) *Biochemistry 11*, 17–23.

Fersht, A. R., Gangloff, J., & Dirheimer, G. (1978) *Biochemistry 17*, 3740–3746.

Gillam, I., Millward, S., Blew, D., Von Tigerstrom, M., Wimmer, E., & Tener, G. M. (1967) *Biochemistry 6*, 3043–3056.

Grosjean, H., Charlier, J., Darte, C., Dirheimer, G., Giegé, R., de Henau, S., Keith, G., Parfait, R., & Takada, C. (1976) *Experientia, Suppl. 26*, 347–362.

Hele, P., & Herbert, L. (1977) *Biochim. Biophys. Acta 479*, 311–321.

Iborra, F., Dorizzi, M., & Labouesse, J. (1973) *Eur. J. Biochem. 39*, 275–282.

Kellermann, O., Brevet, A., Tonetti, H., & Waller, J. P. (1979) *Eur. J. Biochem. 99*, 541–550.

Kern, D., Giegé, R., Robbe-Saul, S., Boulanger, Y., & Ebel, J. P. (1975) *Biochimie 57*, 1167–1176.

Kern, D., Dietrich, A., Fasiolo, F., Renaud, M., Giegé, R., & Ebel, J. P. (1977) *Biochimie 59*, 453–462.

Kern, D., Potier, S., Boulanger, Y., & Lapointe, J. (1979) *J. Biol. Chem. 254*, 518–524.

Kern, D., Zaccaï, G., & Giegé, R. (1980) *Biochemistry 19*, 3158–3164.

Kisselev, L. L., & Favorova, O. O. (1974) *Adv. Enzymol. 40*, 141–238.

Lowry, O. H., Rosebrough, H. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Ostrem, D. L., & Berg, P. (1974) *Biochemistry 13*, 1338–1348.

Pearson, R. L., Weiss, J. F., & Kelmers, A. D. (1971) *Biochim. Biophys. Acta 228*, 770–774.

Pringle, J. R. (1975) *Methods Cell Biol. 12*, 149–184.

Schimmel, P. R., & Söll, D. (1979) *Annu. Rev. Biochem. 48*, 602–648.

Shapiro, A. L., Vinuela, E., & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun. 28*, 815–820.

Som, K., & Hardesty, L. (1975) *Arch. Biochem. Biophys. 166*, 507–517.

Surguchov, A. P., & Surguchova, I. G. (1975) *Eur. J. Biochem. 54*, 175–184.

Vennegor, C., Stols, A. L., & Bloemendal, H. (1972) *J. Mol. Biol. 65*, 375–378.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Wright, H. T., Manor, P. C., Beurling, K., Karpel, R. L., & Fresco, J. L. (1979) *Cold Spring Harbor Monogr. Ser. 9A*, 145–160.