Lund, P. (1985) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. VIII, p 357, Verlag Chemie, Weinheim, FGR.
Mantsala, P., & Zalkin, H. (1984) *J. Biol. Chem. 259*, 14230.
Messenger, L. J., & Zalkin, H. (1979) *J. Biol. Chem. 254*, 3382.
Michal, G. (1984) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. VI, p 191, Verlag Chemie, Weinheim, FGR.
Miyagi, T., & Tsukui, S. (1971) *Biochim. Biophys. Acta 250*, 51.
Miyagi, T., & Tsukui, S. (1979) *Cancer Res. 39*, 2783.
Pinkus, L. M. (1977) *Methods Enzymol. 46*, 414.
Riddles, P. W., Blakeley, R. L., & Zerner, B. (1983) *Methods Enzymol. 91*, 49.
Riou, G. F., Gabillot, M., Barrois, M., Breitburd, F., & Orth, G. (1985) *Eur. J. Biochem. 146*, 483.
Seydoux, F., Malhotra, O. P., & Bernard, S. A. (1974) *CRC Crit. Rev. Biochem. 2*, 227.
Shijo, I., & Ishi, K. (1969) *J. Biochem. (Tokyo) 66*, 175.
Tso, J. Y., Hermodson, M. A., & Zalkin, H. (1982a) *J. Biol. Chem. 257*, 3532.
Tso, J. Y., Zalkin, H., van Cleemput, M., Yanofsky, C., & Smith, J. M. (1982b) *J. Biol. Chem. 257*, 3525.
Vesterberg, O. (1971) *Biochim. Biophys. Acta 243*, 345.
Vollmer, S. J., Switzer, R. L., Hermodson, M. A., Bower, S. G., & Zalkin, H. (1983) *J. Biol. Chem. 258*, 10582.
Walker, J. E., Gay, J., Saraste, M., & Eberle, N. (1984) *Biochem. J. 224*, 799.
Weygand, F., & Reiher, M. (1955) *Chem. Ber. 88*, 26.
Weygand, F., & Geiger, R. (1957) *Chem. Ber. 90*, 634.
Wilkinson, M., Iacobucci, G. A., & Myers, D. V. (1976) *Anal. Biochem. 70*, 470.
Winterburn, P. J., & Phelps, C. F. (1971) *Biochem. J. 121*, 701.
Wong, J. Y., Meyer, E., & Switzer, R. L. (1977) *J. Biol. Chem. 252*, 7424.
Zalkin, H. (1985) *Methods Enzymol. 113*, 278.

Dissociation of the Octameric Bifunctional Enzyme Formiminotransferase–Cyclodeaminase in Urea. Isolation of Two Monofunctional Dimers†

Wendy A. Findlay and Robert E. MacKenzie*
Department of Biochemistry, McGill University, Montreal, Quebec, H3G 1Y6 Canada
Received June 19, 1986; Revised Manuscript Received November 7, 1986

ABSTRACT: Partial denaturation of the circular octameric bifunctional enzyme formiminotransferase–cyclodeaminase in increasing urea concentrations leads to sequential dissociation via dimers to inactive monomers. In potassium phosphate buffer, dissociation to dimers in 3 M urea coincides with loss of both activities and a major decrease in intensity of intrinsic tryptophan fluorescence. In the presence of folic acid, these dimers retain the deaminase activity, but with folylpolyglutamates, both activities are protected and the native octameric structure is retained. The protection profiles with polyglutamates are cooperative with a Hill coefficient greater than 2, suggesting that binding of more than one folylpolyglutamate per octamer is required to stabilize the native structure. In triethanolamine hydrochloride buffer, transferase-active dimers that retain the intrinsic tryptophan fluorescence can be obtained in 1 M urea and stabilized at higher urea concentration by the addition of glutamate. Deaminase-active dimers are obtained by the protection of folate in 3 M urea. Proteolysis of the two kinds of dimers by chymotrypsin leads to very different fragmentation patterns, indicating that they are structurally different. We propose that the two dimers retain different subunit–subunit interfaces, one of which is required for each activity. This suggests that the native octameric structure is required for expression of both activities and therefore for "channeling" of intermediates.

The folate-dependent bifunctional enzyme formiminotetrahydrofolate:glutamate formiminotransferase (EC 2.1.2.5)–formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) catalyzes two sequential reactions of the histidine degradation pathway in mammalian liver:

formiminoglutamate + $H_4PteGlu_n$ ⇌ formimino-$H_4PteGlu_n$ + glutamate

formimino-$H_4PteGlu_n$ ⇌ methenyl-$H_4PteGlu_n$ + NH_4^+

With longer polyglutamate derivatives $H_4PteGlu_n$ ($n \geq 4$)[1] direct transfer of the formimino intermediate from the transferase site to the deaminase site has been observed, with complete "channeling" of the pentaglutamate (Paquin et al., 1985). The channeling requires noncovalent attachment of the intermediate to the enzyme, possibly to a separate polyglutamate binding site that "anchors" the substrate while the pteroyl moiety is transferred from the transferase site to the deaminase catalytic site (MacKenzie & Baugh, 1980). There appears to be a steric requirement for optimal channeling since the efficiency of channeling does not correlate directly with either binding affinity or kinetic efficiency (V_m/K_m) as polyglutamate chain length increases (Paquin et al., 1985).

† Supported by a grant from the Medical Research Council of Canada.

[1] Abbreviations: $H_4PteGlu_n$, tetrahydropteroylpolyglutamate with n glutamates; PteGlu, folic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TEA·HCl, triethanolamine hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

0006-2960/87/0426-1948$01.50/0

When isolated from pig liver, the enzyme is composed of eight identical polypeptides of M_r 62 000 arranged in a ring, as observed by electron microscopy (Beaudet & MacKenzie, 1976). Cross-linking experiments with 1,5-difluoro-2,4-dinitrobenzene indicated two types of subunit interaction, suggesting a tetramer of dimers structure which was confirmed by the production of a dimeric transferase-active fragment on treatment of the enzyme with chymotrypsin (MacKenzie et al., 1980).

Paquin et al. (1985) have observed only four high-affinity sites for binding of tetrahydropteroylpolyglutamates to the native octamer, presumably one site per dimer. Kinetic experiments suggest that the catalytic sites can function independently but that there is a single polyglutamate binding site per pair of transferase/deaminase sites. The existence of only four polyglutamate binding sites suggests that the dimer may be the basic functional unit of this enzyme and that the sites may be formed by one of the two kinds of subunit–subunit interactions. The role of the unusual quaternary structure of formiminotransferase–cyclodeaminase in its ability to channel intermediate has not been determined.

In this paper, we present results showing that partial denaturation at low urea concentrations dissociates the octameric enzyme to produce two active dimeric species. The isolation and characterization of these dimers helps to define the role of the quaternary structure in enzyme activity and provides evidence for site formation by each type of subunit–subunit association.

Materials and Methods

The formiminotransferase–cyclodeaminase enzyme was prepared and assayed as described previously (Drury et al., 1975). Preparations routinely had a transferase specific activity of 41 μmol min^{-1} mg^{-1} and yielded a single band on SDS–PAGE. The transferase-active fragment was generated by chymotryptic cleavage of the enzyme in the presence of folate (MacKenzie et al., 1980); it had a specific activity of 29 μmol min^{-1} mg^{-1} and also yielded a single band on SDS–PAGE after purification by gel filtration.

Ultrapure urea was purchased from Canadian Scientific Products, folic acid and formimino-L-glutamic acid were from Sigma Chemical Co., and bis(sulfosuccinimidyl) suberate was from Pierce Chemical Co. Pteroylpolyglutamates were a gift from Dr. Charles Baugh, University of South Alabama. Other chemicals were reagent grade: Tween 80, triethanolamine, and EDTA from Fisher Scientific, sodium and potassium phosphates from J. T. Baker Chemical Co., 2-mercaptoethanol from Kodak, and DTT from Boehringer-Mannheim. (6*RS*)-H_4PteGlu was prepared chemically and purified as described previously (Drury et al., 1975).

Assays. The transferase assays were performed as reported by Drury et al. (1975) but with use of a 0.5-mL incubation volume and measurement of A_{350} on a Bausch & Lomb Spectronic 2000 spectrophotometer. Deaminase assays contained 60 μM (6*S*)-formimino-H_4PteGlu in 0.5 mL of 0.1 M potassium phosphate (pH 7.3) and 35 mM 2-mercaptoethanol. The appearance of (6*R*)-5,10-methenyl-H_4PteGlu was monitored at 355 nm (ϵ = 24 900) with either a Spectronic 2000 or a Beckman DU-7 spectrophotometer after addition of 10 μL (50 ng) of enzyme. The (6*S*)-formimino-H_4PteGlu was prepared from 1.8 mM (6*RS*)-H_4PteGlu by using the transferase fragment, as described by Paquin et al. (1985). All activity measurements are the average of assays done in duplicate or triplicate.

Denaturation. Stock enzyme solution [2 mg/mL in 0.1 M potassium phosphate (pH 7.3), 40% glycerol, 35 mM 2-mercaptoethanol] was diluted 400-fold into 0.1 M potassium phosphate (pH 7.3) or 0.1 M TEA·HCl (pH 7.3) buffer containing 35 mM 2-mercaptoethanol, 0.05% Tween 80, and the required urea concentration. After 2 h at room temperature the two activities were assayed. Control experiments showed that the enzyme was stable under these conditions in the absence of urea, and time courses in the presence of urea indicated that the major decrease in activity occurs in the first 2 h, followed by a slower loss thereafter. For the protection experiments, the substrate analogues and the enzyme were added to the buffer 15 min before addition of an equal volume of urea in the same buffer and then incubated for 2 h at room temperature before assay. The experiments with transferase fragment were performed in the same manner, except that only a 20-fold dilution was used from a stock solution containing 102 μg/mL.

Fluorescence. A Perkin-Elmer LS-5 fluorescence spectrophotometer was used with excitation at 290 nm and the emission scanned from 320 to 400 nm to determine the wavelength and peak height of maximum emission. Slit widths of 3 mm were used for both excitation and emission, and samples were prepared exactly as for the denaturation experiments.

Cross-Linking and Electrophoresis. A 10 mg/mL solution of bis(sulfosuccinimidyl) suberate was freshly prepared in 0.05 M sodium phosphate (pH 7.4), and 30-μL aliquots were added to 20 μg of formiminotransferase–cyclodeaminase or 10 μg of fragment that had been incubated for 2 h in 0.5 mL of 0.1 M potassium phosphate (pH 7.3) or 0.1 M TEA·HCl (pH 7.3), 0.05% Tween 80, 1 mM DTT, and 1 mM EDTA, containing the required urea concentration and protective agents (as indicated in the figure legends). After 20 min, 55 μL of 20 mg/mL lysine was added and left for 10 min to react with excess cross-linker. The samples were precipitated by the method of Bensadoun and Weinstein (1976). For electrophoresis, the phosphate system of Weber and Osborn (1969) was used with samples redissolved in 50 μL of dissolving buffer, loaded on 4% polyacrylamide tube gels, and electrophoresed at 6–8 mA per gel for ~4 h. For experiments with the transferase fragment, 5% polyacrylamide gels were used.

Limited Proteolysis. Enzyme (15 μg in 15 μL) was added to 400 μL of 0.1 M TEA·HCl (pH 7.3), 35 mM 2-mercaptoethanol, and 0.05% Tween containing the required protecting agent (20 mM glutamate or 1 mM PteGlu). After 10 min, 400 μL of urea solution in the same buffer was added to give the required final urea concentration. Samples were incubated for 2 h at room temperature and then assayed for both activities. Prior to proteolysis, each sample was diluted to contain 2 M urea and both protecting agents, glutamate and PteGlu. To the final volume of 1.2 mL was added 20 μL of 40 μg/mL chymotrypsin in 1 mM HCl. The samples were left for 1 h at room temperature, and then 20 μL of 2.5 mg/mL phenylmethanesulfonyl fluoride in 30% 2-propanol was added to stop proteolysis. After another 10 min at room temperature, the samples were cooled on ice and cold 100% (w/v) trichloroacetic acid was added to give a final concentration of 20% (w/v) TCA. After 15 min on ice, the samples were centrifuged at 13000*g* in a Fisher microcentrifuge for 15 min, and the supernatant was removed. The pellets were washed with 250 μL of cold 2% (w/v) trichloroacetic acid, and the tubes were centrifuged again for 15 min. The pellets were resuspended in 50 μL of sample buffer for electrophoresis using the discontinuous SDS system of Laemmli (1970) with a 12% resolving gel and a 3% stacking gel.

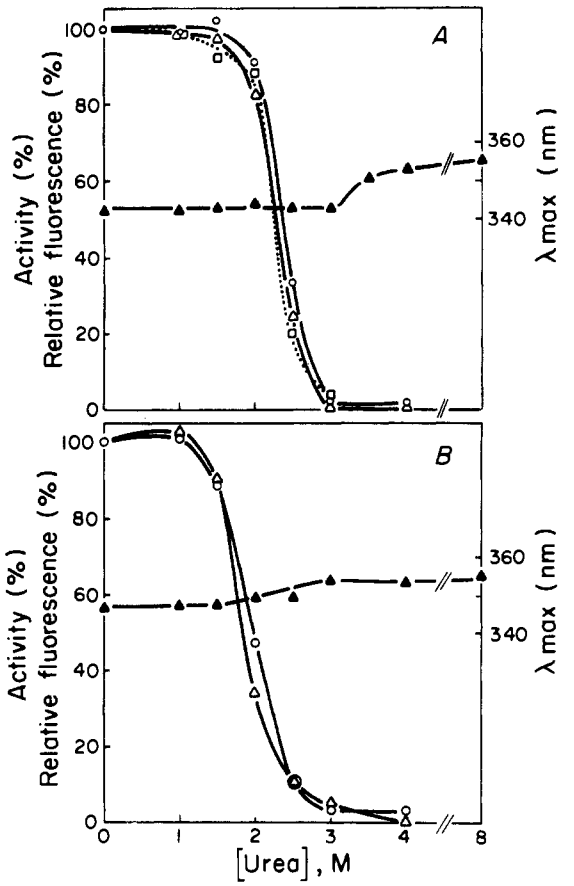


FIGURE 1: (A) Inactivation and intrinsic tryptophan fluorescence of formiminotransferase–cyclodeaminase in different urea concentrations. Final concentrations were 10 nM enzyme in 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, and 0.05% Tween 80. (B) Inactivation and intrinsic tryptophan fluorescence of a proteolytically derived dimeric transferase fragment in different urea concentrations. Final concentrations were 65 nM dimeric fragment in 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween 80, and indicated urea concentration. Symbols: (○) percent original transferase activity; (□) percent original deaminase activity; (△) percent relative fluorescence = $(F - F_4)/(F_0 - F_4)$, where F is maximum peak height, F_0 is fluorescence in the absence of urea, and F_4 is fluorescence in 4 M urea; (▲) wavelength of maximum emission.

RESULTS

Inactivation in Urea and Protection by Substrate Analogues. In potassium phosphate buffer both activities of the bifunctional enzyme decrease simultaneously as the urea concentration is increased from 2 to 3 M (Figure 1A). As has been found in other systems (Ghélis & Yon, 1982), the inactivation profile is highly cooperative.

Table I: Parameters for Fitting Protection Profiles by Hill Equation[a]

substrate analogue	activity	maximum protection (%)	concn at half-maximum (μM)	h
$PteGlu_5$	transferase	44 ± 2	10.4 ± 0.5	2.5 ± 0.2
	deaminase	67 ± 7	12 ± 2	1.8 ± 0.2
$PteGlu_3$	transferase	45 ± 1	60 ± 2	2.3 ± 0.1
	deaminase	62 ± 1	48 ± 1	2.3 ± 0.1
$PteGlu_1$	transferase	≤5		
	deaminase	50 ± 1	154 ± 4	1.15 ± 0.02

[a] Parameters and standard deviations were generated by computer fits of the data in Figures 1B and 2A,B.

In an attempt to differentiate between the two catalytic sites, addition of substrate analogues was used to protect against the effects of 3 M urea. Folic acid was found to protect only the deaminase activity (Figure 2A) and exerted its half-maximal effect at 160 μM. Although the values of K_i determined kinetically for folate against the transferase activity (44 μM) and the deaminase activity (50 μM) are similar (J. Paquin, unpublished results), no protection of the transferase activity was observed with up to 500 μM folic acid. Both activities were protected by the addition of pteroylpolyglutamates. In the presence of the pentaglutamate, which is the optimal length for substrate channeling (Paquin et al., 1985), substantial fractions of both activities were retained (Figure 2C). Half-maximal protection is observed at ~12 μM $PteGlu_5$, and the initial portions of the two curves are similar with divergence at higher $PteGlu_5$ concentrations. Pteroyltriglutamate, whose tetrahydro derivative is not channeled by the enzyme, also protected both activities, but the two curves are more divergent (Figure 2B) with less protection of the transferase activity. Half-maximal protection occurred with 50–60 μM $PteGlu_3$, and the 3-fold decrease from the value for folic acid agrees well with the 3-fold decrease in dissociation constant for the triglutamate vs. monoglutamate derivative of tetrahydrofolate for this enzyme (Paquin et al., 1985).

The extent of protection as a function of the concentration of each of the polyglutamates indicates that the effect is very cooperative, and a computer fit of the data to the Hill equation using the program Kinfit (Knack & Röhm, 1981) yielded the parameters shown in Table I. These values were used to generate the curves in Figure 2, with the residual activities in the absence of folates (~10% for deaminase and ~3% for

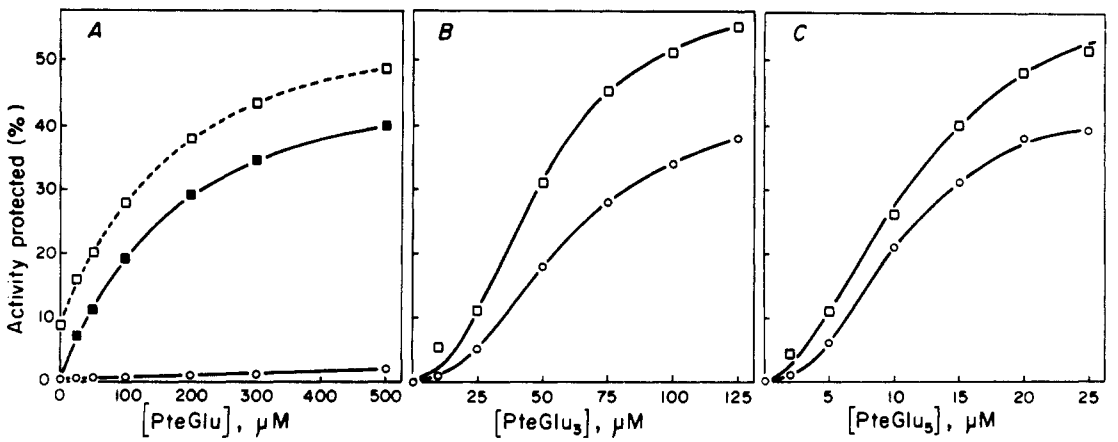


FIGURE 2: Protection of enzyme activities in 3 M urea by substrate analogues. Activities as a function of concentration of (A) PteGlu, (B) $PteGlu_3$, and (C) $PteGlu_5$, with 10 nM enzyme in 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween 80 and 3 M urea. Symbols: (○) transferase activity, (□) deaminase activity, and (■) with initial value subtracted.

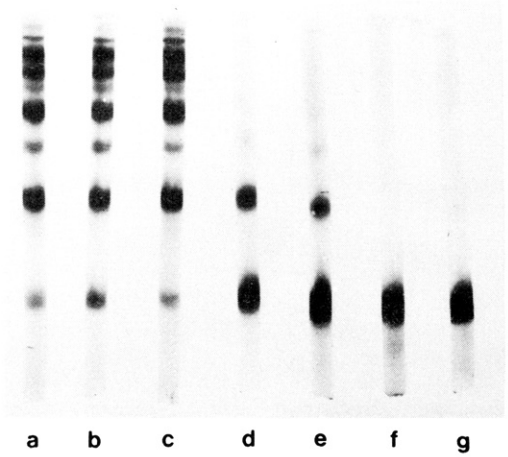


FIGURE 3: SDS–PAGE analysis (Weber & Osborn, 1969) of formiminotransferase–cyclodeaminase cross-linked in different urea concentrations. Each gel has 20 μg of enzyme cross-linked with 0.55 mg/mL bis(sulfosuccinimidyl) suberate in 0.1 M potassium phosphate, 1 mM DTT, 1 mM EDTA, 0.05% Tween 80, (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, or (f) 8 M urea, and (g) no cross-linking.

transferase) subtracted. The average value of the Hill coefficient was 2.2 for polyglutamates but was close to 1 for folate itself.

Changes in Protein Fluorescence. The intensity of the intrinsic tryptophan fluorescence was found to decrease concomitantly with the loss of activity over the same range of urea concentration (Figure 1A), reflecting a corresponding physical change in the structure of the enzyme between 2 and 3 M urea. The coincidence between the profiles of inactivation and decrease in fluorescence intensity suggests a two-state process, possibly either subunit dissociation or a major conformational change. At higher urea concentrations (between 3 and 4 M) a red shift in λ_{max} of fluorescence emission is observed, corresponding to a second physical change in the enzyme, further exposing the tryptophan residues.

The transferase fragment also shows coincidence of the deactivation profile in urea and the decrease in intensity of intrinsic tryptophan fluorescence (Figure 1B). This transition occurs at a lower urea concentration (1.5–2.5 M), suggesting that the dimeric fragment is less stable than the native octamer. A smaller change in λ_{max} of fluorescence emission was observed over the same range of urea concentration as the loss of activity, so the separate physical changes observed during denaturation of the enzyme occur together in the fragment.

Cross-Linking. The hydrophilic bifunctional reagent bis(sulfosuccinimidyl) suberate, which reacts primarily with lysine groups, was found to give good cross-linking of the enzyme under the experimental conditions. It resulted in production of substantial amounts of all species up to octamer as shown in lane a of Figure 3, and a plot of log M_r (or number of subunits) vs. R_f was linear from dimer to hexamer (not shown). Two bands are observed for the octamer, probably corresponding to linear and circular forms. Darker bands corresponding to dimer, tetramer, hexamer, and octamer confirm the tetramer of dimers structure.

Cross-linking of the enzyme in the presence of urea indicated that the loss of activity corresponded to a change in the quaternary structure. As shown in Figure 3, little difference in the cross-linking profile is observed in 0, 1, and 2 M urea. In 3 M urea there is a drastic reduction in the amount of species higher than dimer, and in 4 M urea a significant amount of dimer is retained, but only monomer is observed in 8 M urea. Dissociation of the octamer in 3 M urea was confirmed by gel filtration on a column of LKB AcA-34 gel,

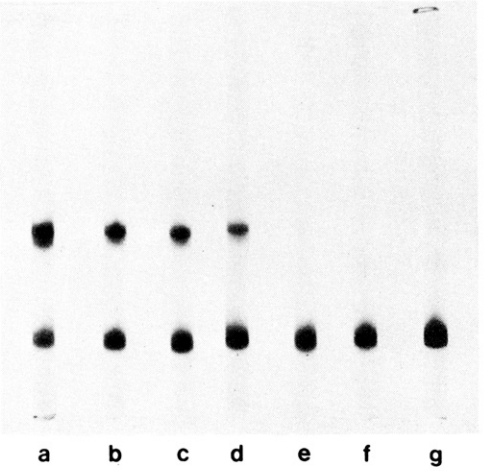


FIGURE 4: SDS–PAGE analysis of transferase fragment cross-linked in different urea concentrations. Each gel has 10 μg of fragment cross-linked with 0.55 mg/mL bis(sulfosuccinimidyl) suberate in 0.1 M potassium phosphate, 1 mM DTT, 1 mM EDTA, 0.05% Tween 80, (a) 0, (b) 1, (c) 1.5, (d) 2, (e) 3, or (f) 4 M urea, and (g) no cross-linking.

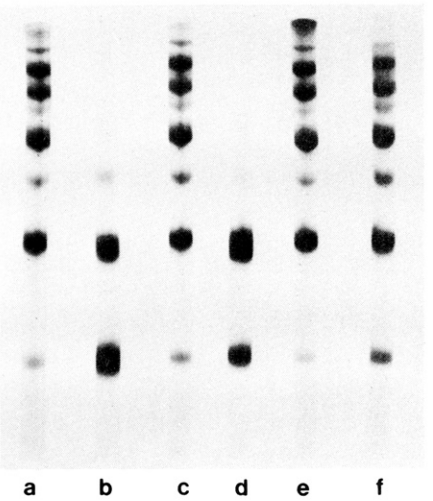


FIGURE 5: SDS–PAGE analysis of formiminotransferase–cyclodeaminase cross-linked in the presence and absence of urea and substrate analogues. Gels have 20 μg of protein cross-linked by 0.55 mg/mL bis(sulfosuccinimidyl) suberate in 0.1 M potassium phosphate, 1 mM EDTA, 1 mM DTT, 0.05% Tween 80, and indicated concentrations of urea and protective agent: (a) no additives, (b) 3 M urea, (c) 500 μM PteGlu, (d) 3 M urea and 500 μM PteGlu, (e) 22 μM $PteGlu_5$, or (f) 3 M urea and 22 μM $PteGlu_5$.

in the presence and absence of 500 μM folic acid.

Bis(sulfosuccinimidyl) suberate also gives reasonably good cross-linking of the transferase fragment, and as shown in Figure 4, loss of activity with increasing urea concentration accompanies dissociation of the dimer.

The presence of 20 μM pteroylpentaglutamate preserves both subunit–subunit interactions of the octameric enzyme in 3 M urea, as indicated by the presence of all species up to octamer after cross-linking (Figure 5). A similar effect on quaternary structure was observed with 125 μM triglutamate (data not shown). The binding of polyglutamates thus seems to strengthen dimer–dimer interactions. However, the addition of 500 μM folic acid increased only the amount of dimer observed after cross-linking in 3 M urea and had little effect on the amount of higher forms. It appears to protect by binding to the deaminase site and may strengthen subunit–subunit interaction within the dimer.

Production of Two Kinds of Dimer by Urea Denaturation in Triethanolamine Hydrochloride Buffer. Formimino-

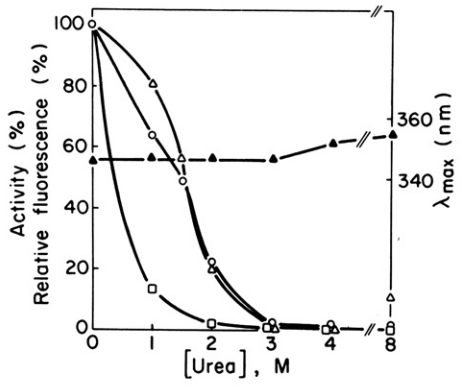


FIGURE 6: Inactivation and intrinsic tryptophan fluorescence of formiminotransferase–cyclodeaminase in TEA·HCl and increasing urea concentration. Final concentration was 10 nM octameric enzyme in 0.1 M TEA·HCl (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween 80, and indicated urea concentration. Symbols: (○) transferase and (□) deaminase activities, (△) relative fluorescence intensity (see Figure 1), and (▲) wavelength of maximum emission.

Table II: Protection by Glutamate of Transferase Activity and Intrinsic Tryptophan Fluorescence of the Enzyme in 2 M Urea[a]

[glutamate] (mM)	transferase activity (%)	fluorescence (%)
0	12	47
0.5	28	56
1	35	61
2	36	69
4	41	76
8	49	83
native enzyme (+8 mM glutamate)	100	100

[a] Addition of glutamate affected fluorescence of native enzyme by <5%. No protection of deaminase activity was observed.

transferase–cyclodeaminase is less stable in TEA·HCl buffer than in potassium phosphate, and under these conditions, the deaminase is inactivated in 1 M urea, while the transferase is preferentially retained, as shown in Figure 6. The loss of transferase activity with increasing urea concentration is less cooperative than that observed in potassium phosphate, and the midpoint of the profile occurs at 1.5 M urea. The loss of intrinsic tryptophan fluorescence intensity corresponds more closely to inactivation of the transferase than that of the deaminase. The red shift of the wavelength of maximum fluorescence emission occurs between 3 and 4 M urea, which is the same as in potassium phosphate.

It is possible to protect the two activities separately against urea denaturation in TEA·HCl buffer. Table II shows protection by glutamate of the transferase activity and of the intrinsic tryptophan fluorescence in 2 M urea. The effect of glutamate is not cooperative and is half-maximal at about 1 mM. The deaminase activity is protected in the presence of folate, even in 3 M urea, as shown in Figure 7. The protection by folate is not cooperative ($h = 1.1 \pm 0.1$), and half-maximal protection occurs at a concentration of 260 μM, which is somewhat higher than the value in potassium phosphate (154 μM). Folylpolyglutamates (≤30 μM) did not protect either activity in 3 M urea.

Cross-linking by bis(sulfosuccinimidyl) suberate in the presence of urea and TEA·HCl indicates that dissociation to dimers occurs between 0 and 1 M urea (Figure 8A). As the urea concentration is increased, dissociation of the dimers occurs, and little dimer is left in 4 M urea. The cross-linking patterns are changed by the addition of the protecting agents, as shown in Figure 8B. In the presence of folic acid, the band corresponding to dimer in the native enzyme is more intense,

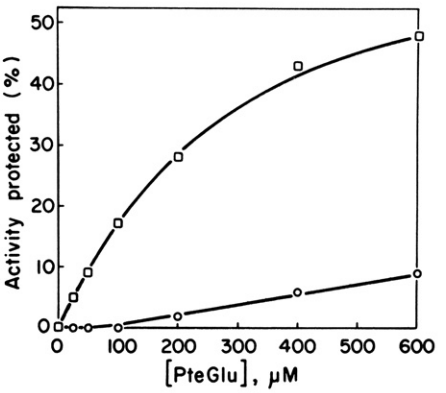


FIGURE 7: Preferential protection of deaminase activity in 3 M urea and TEA·HCl by PteGlu. Final concentration was 10 nM octameric enzyme in 0.1 M TEA·HCl (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween 80, 3 M urea, and indicated PteGlu concentration. Symbols: (□) deaminase and (○) transferase activities.

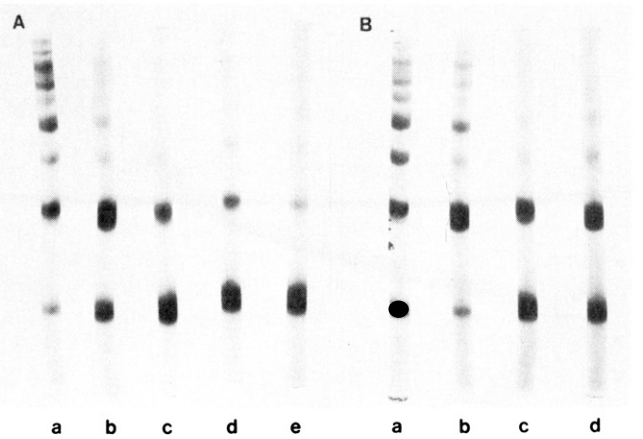


FIGURE 8: SDS–PAGE analysis of formiminotransferase–cyclodeaminase cross-linked in different urea concentrations and in the presence of substrate analogues. Each gel has 20 μg of enzyme cross-linked with 0.55 mg/mL bis(sulfosuccinimidyl) suberate in 0.1 M TEA·HCl, 1 mM DTT, 1 mM EDTA, and 0.05% Tween 80. (A) Effect of increasing urea concentration: (a) 0, (b) 1, (c) 2, (d) 3, and (e) 4 M urea. (B) Effect of protecting agents: (a) 0 M urea and 10 mM glutamate, (b) 0 M urea and 500 μM PteGlu, (c) 2 M urea and 10 mM glutamate, and (d) 3 M urea and 500 μM PteGlu.

and in 3 M urea a marked increase in the amount of dimer is observed. The addition of glutamate to protect the transferase activity in 2 M urea also appears to increase the amount of dimer present. In both cases, the amount of higher forms is not increased.

The production of monofunctional dimers retaining either the transferase or deaminase activity raised the possibility that different subunit–subunit interfaces had been isolated from the native tetramer of dimers structure. The sensitivities of the two dimers to proteolysis by chymotrypsin supported this, since quite different peptides were obtained on SDS–PAGE (Figure 9). Digestion of the transferase-active dimer gave results similar to those for the native enzyme, two major bands at M_r 39 000 and 24 000. The deaminase-active dimer showed a much different peptide pattern, without the production of the M_r 39 000 species. The deaminase activity is lost during proteolysis, whereas the transferase activity is retained by both the transferase-active dimer and the native enzyme (Table III).

Discussion

Most intracellular proteins occur as oligomers, with dimers and tetramers being by far the most common. If the subunits are identical, the interactions between them can be either homologous or heterologous, yielding different symmetries in

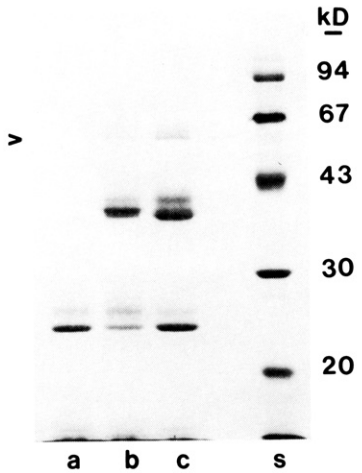


FIGURE 9: SDS–PAGE analysis (Laemmli, 1970) of products of limited proteolysis of two kinds of dimer. The first three lanes contain 15 μg of enzyme treated with 0.67 μg/mL chymotrypsin under the conditions described under Materials and Methods: (a) deaminase dimer produced in 3 M urea and 500 μM PteGlu, (b) transferase dimer produced in 2 M urea and 10 mM glutamate, and (c) native enzyme incubated with 500 μM PteGlu and 10 mM glutamate. Lane s contains Pharmacia low molecular weight standards. The arrowhead indicates the position of uncleaved subunit.

Table III: Activities before and after Proteolysis of Transferase- and Deaminase-Active Dimers by Chymotrypsin

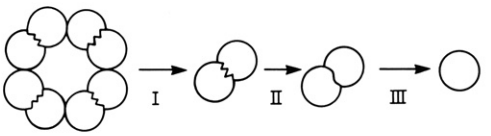
	activity before proteolysis (%)		activity after proteolysis (%)	
enzyme form	transferase	deaminase	transferase	deaminase
native octamer	100	100	70	5
transferase dimer	58	4	54	≤1
deaminase dimer	5	62	≤1	2

the quaternary structure (Ghelis & Yon, 1982). These interactions are mainly hydrophobic and serve to increase the stability of the protein, so arrangements maximizing subunit contacts are preferred. Methods of studying the folding and association of proteins have been reviewed by Jaenicke (1982), and most results show that folded monomers are inactive and that full enzymatic activity requires subunit association.

The unusual circular octameric structure of formimino-transferase–cyclodeaminase, combined with its bifunctional nature and its ability to channel noncovalently bound polyglutamate substrates between active sites, makes this enzyme a particularly suitable system for studying site–site interactions and the relationship of activity to quaternary structure. Binding studies and kinetic experiments suggested that the dimer may be the basic functional unit of this enzyme, with a single polyglutamate binding site per pair of transferase/deaminase sites (Paquin et al., 1985). In this study we have used low concentrations of urea to dissociate the bifunctional octameric enzyme. Assays for both activities, intrinsic tryptophan fluorescence, ligand-induced stabilization, and cross-linking of species to determine quaternary structure were used to identify intermediates.

Dissociation by Urea in Phosphate Buffer. Using partial denaturation by urea in potassium phosphate buffer, we were able to observe three separate transitions, for which we propose Scheme I. The first transition (I) occurs as the urea concentration is increased from 2 to 3 M and is observed as a simultaneous loss of both activities (in the absence of folate) accompanied by a decrease in intensity of the intrinsic tryptophan fluorescence and dissociation of the octameric enzyme to dimers. Transition II corresponds to a physical change in these dimers between 3 and 4 M urea and is observed as a red

Scheme I



shift in the wavelength of maximum fluorescence emission. At urea concentrations above 4 M, dissociation of the dimers to monomers constitutes the third transition (III). In the presence of folic acid, transition I generates monofunctional deaminase-active dimers.

The dimeric transferase-active fragment, which isolates one type of subunit–subunit interaction, undergoes a single transition, with the loss of activity in urea accompanied by a decrease in intensity of tryptophan fluorescence and by dissociation of the dimer to monomers. We propose that the interface isolated in the dimeric transferase fragment is the one that is disrupted in transition I, since in both cases a substantial decrease in intensity of intrinsic tryptophan fluorescence and complete loss of transferase activity accompany the dissociation.

The presence of substrate analogues protected the enzyme from inactivation in 3 M urea. The marked differential protection of the two activities by folate and pteroyltriglutamate confirms that the active sites are separate, as previously indicated by chemical modification (Drury & MacKenzie, 1977; MacKenzie & Baugh, 1980) and kinetic studies (Paquin et al., 1985). The protection profile for folate is not cooperative ($h \simeq 1$), suggesting that it binds directly to the deaminase catalytic site, stabilizing the portion of the polypeptide responsible for this activity and possibly strengthening subunit–subunit interaction within the dimer. Protection of the two activities by pteroylpentaglutamate may be related to the ability of the enzyme to channel the pentaglutamate substrate completely, if it binds to a separate polyglutamate site and the tetrahydropteroyl portion can reach both active sites. This does not necessarily imply that the two active sites are close together, since the distance from the N-5 position of the tetrahydropteroyl ring to the α-carboxyl group of the tightly binding fourth glutamate is calculated to be 20–25 Å (Paquin et al., 1985). Binding of polyglutamates appears to strengthen the dimer–dimer interactions, and the apparent cooperativity ($h \geq 2$) may be explained if the four polyglutamate-binding sites per octamer observed by Paquin et al. (1985) are located between dimers and if binding to two or more sites is required to stabilize the octameric structure.

Production of Two Types of Dimer in Triethanolamine Hydrochloride. The native transferase–deaminase is stabilized or activated by both monovalent cations and polyvalent anions (Tabor & Wyngarden, 1959; Drury et al., 1975) and is clearly less stable in the absence of potassium and phosphate. Dissociation of the enzyme by urea in TEA·HCl buffer proceeds by a different sequence from that seen in potassium phosphate, with dissociation to dimers and preferential retention of the transferase activity in only 1 M urea. As the concentration of urea is increased from 1 to 3 M, dissociation to monomers, loss of transferase activity, and a decrease in the intrinsic tryptophan fluorescence of the protein all occur. The transferase dimer produced in low concentrations of urea retains the native fluorescence, which is lost upon further dissociation, and the addition of glutamate protected both the transferase activity and the intrinsic tryptophan fluorescence at higher urea concentration. As found for the studies in phosphate buffer, the deaminase activity was preferentially stabilized by folic acid, and in 3 M urea dimers with only the deaminase activity

were produced in the presence of folate.

The formation of the two monofunctional dimers appears to involve dissociation of different subunit–subunit interactions. This is supported by the association in all cases of fluorescence changes and transferase activity with a single interface, as well as by the very different stabilities of the two dimers to urea. Further evidence is provided by limited proteolysis of the two dimers by chymotrypsin. The domain responsible for transferase activity (M_r 39 000) is protected in both the native enzyme and the transferase dimer but is completely digested in the deaminase dimer, as expected if different portions of the polypeptide are protected by contact between subunits.

For several enzymes, the requirement of subunit association for activity has been shown to be due to the formation of active sites at the interfaces between subunits, with essential residues contributed by both polypeptides. Catalytic sites can be formed by either isologous (Ford et al., 1980; Thieme et al., 1981) or heterologous (Krause et al., 1985; Almassy et al., 1986) interactions. The physical location of the two catalytic sites of formiminotransferase–cyclodeaminase and their relationship to the polyglutamate binding site will only be resolved by X-ray crystallography of this enzyme.

The results presented here suggest that the transferase and deaminase activities require the integrity of alternate subunit–subunit interfaces. This indicates that dimers are not the basic functional unit of this enzyme and that the octameric structure is required for simultaneous expression of both activities and therefore is essential for substrate channeling.

ACKNOWLEDGMENTS

We thank Leonora Bortoluzzi for preparation of the enzyme used for preliminary experiments, Maureen Caron for typing the manuscript, and Kathy Teng for the photography.

REFERENCES

Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N.-H., & Eisenberg, D. (1986) *Nature (London) 323*, 304–309.

Beaudet, R., & MacKenzie, R. E. (1976) *Biochim. Biophys. Acta 453*, 151–161.

Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.

Drury, E. J., & MacKenzie, R. E. (1977) *Can. J. Biochem. 55*, 919–923.

Drury, E. J., Bazar, L. S., & MacKenzie, R. E. (1975) *Arch. Biochem. Biophys. 169*, 662–668.

Ford, G. C., Eichele, G., & Jansonius, J. N. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2559–2563.

Ghélis, C., & Yon, J. (1982) in *Protein Folding*, pp 232–238, Academic, New York.

Jaenicke, R. (1982) *Biophys. Struct. Mech. 8*, 231–256.

Knack, I., & Röhm, K.-H. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1119–1130.

Krause, K. L., Volz, K. W., & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1643–1647.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

MacKenzie, R. E., & Baugh, C. M. (1980) *Biochim. Biophys. Acta 611*, 187–195.

MacKenzie, R. E., Aldridge, M., & Paquin, J. (1980) *J. Biol. Chem. 255*, 9474–9478.

Paquin, J., Baugh, C. M., & MacKenzie, R. E. (1985) *J. Biol. Chem. 260*, 14925–14931.

Tabor, H., & Wyngarden, L. (1959) *J. Biol. Chem. 234*, 1830–1846.

Thieme, R., Pai, E. F., Schirmer, R. H., & Schulz, G. E. (1981) *J. Mol. Biol. 152*, 763–782.

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