is given on any current masthead page.

REFERENCES

Bell, R. P. (1966) *Adv. Phys. Org. Chem. 4*, 1.
Brodbeck, V., Schweikert, K., Gentinetta, R., & Rottenberg, M. (1979) *Biochim. Biophys. Acta 567*, 357.
Bush, K., Henry, P. R., & Slusarchyk, D. S. (1984) *J. Antibiot. 37*, 330.
Chen, R., Gorenstein, D. G., Kennedy, W. P., Lowe, G., Nurse, D., & Schultz, R. M. (1979) *Biochemistry 18*, 921.
Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., & Cushman, D. W. (1980) *J. Biol. Chem. 255*, 401.
Ellman, G. L., Courtney, D. K., Andres, V., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88.
Folk, J. E., & Schirmer, E. W. (1963) *J. Biol. Chem. 238*, 3884.
Fruton, J. S. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 1.
Galardy, R. E., & Kortylewicz, Z. P. (1984) *Biochemistry 23*, 2083.
Guthrie, J. P. (1975) *Can. J. Chem. 53*, 898.
Hammock, B. D., Wing, K. D., McLaughlin, J., Lovell, V. M., & Sparks, T. C. (1982) *Pestic. Biochem. Physiol. 17*, 76.
Hine, J., & Redding, R. W. (1970) *J. Org. Chem. 35*, 2769.
Holmquist, B., Bunning, P., & Riordan, J. F. (1979) *Anal. Biochem. 95*, 540.
Lowe, G., & Nurse, D. (1977) *J. Chem. Soc., Chem. Commun.*, 815.
Marciniszyn, J., Hartsuck, J. A., & Tang, J. (1976) *J. Biol. Chem. 251*, 7088.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527.
Petrillo, E. W., Jr., & Ondetti, M. A. (1982) *Med. Res. Rev. 2*, 1.
Prestwich, G. D., Eng, W.-S., Roe, R. M., & Hammock, B. D. (1984) *Arch. Biochem. Biophys. 228*, 639.
Rich, D. H., Sun, E. T. O., & Ulm, E. (1980) *J. Med. Chem. 23*, 27.
Rich, D. H., Boparai, A. S., & Bernatowicz, M. S. (1982a) *Biochem. Biophys. Res. Commun. 104*, 3535.
Rich, D. H., Bernatowicz, M. S., & Schmidt, P. G. (1982b) *J. Am. Chem. Soc. 104*, 3535.
Rich, D. H., Salituro, F. G., Holladay, M. W., & Schmidt, P. S. (1984) in *Conformationally Directed Drug Design* (Vida, J. A., & Gordon, M., Eds.) ACS Symp. Ser. No. 251, pp 211–237, American Chemical Society, Washington, DC.
Shah, D. O., Lai, K., & Gorenstein, D. G. (1984) *J. Am. Chem. Soc. 106*, 4272.
Sugimoto, T., & Kaiser, E. T. (1978) *J. Am. Chem. Soc. 100*, 7750.
Thompson, R. C. (1973) *Biochemistry 12*, 47.
Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Hamada, M., & Takevehi, T. (1970) *J. Antibiot. (Tokyo) 23*, 259.
Westerik, J. O., & Wolfenden, R. (1971) *J. Biol. Chem. 247*, 8195.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.
Wolfenden, R. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 271.
Workman, R. J., & Burkitt, D. S. (1977) *Arch. Biochem. Biophys. 194*, 157.

Articles

Evidence for Active Intermediates during the Reconstitution of Yeast Phosphoglycerate Mutase

Reinhard Hermann[‡] and Rainer Jaenicke*

Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-8400 Regensburg, FRG

Nicholas C. Price[§]

Department of Biological Science, University of Stirling, Stirling FK9 4LA, Scotland, U.K.

Received September 4, 1984

ABSTRACT: The reconstitution of the tetrameric phosphoglycerate mutase from bakers' yeast after denaturation in guanidine hydrochloride has been studied. When assays are performed in the presence of trypsin, it is found that reactivation parallels the regain of tetrameric structure. However, in the absence of trypsin, the regain of activity is more rapid, suggesting that monomeric and dimeric intermediates possess partial activity (35% of the value of native enzyme) which is sensitive to trypsin. When reconstitution is studied in the presence of substrates, it is again found that monomeric and dimeric intermediates possess 35% activity. Under these latter conditions, the activity of the monomer but not of the dimer is sensitive to trypsin.

The reconstitution of oligomeric enzymes after denaturation has become a useful experimental model for the folding and association of these enzymes during biosynthesis [for a review, see Jaenicke (1984)]. Comparative studies of the rates of regain of enzyme activity and of quaternary structure, e.g., by use of the glutaraldehyde cross-linking technique (Hermann et al., 1981), have allowed the kinetic aspects of the pathway of reconstitution to be explored for a number of enzymes and permitted deductions to be drawn regarding the catalytic properties of intermediate species. In most cases, it appears

[‡] Present address: Behringwerke AG, Forschung Biochemie/Immunologie, D-3550 Marburg, FRG.

[§] Recipient of a fellowship from the Alexander von Humboldt Stiftung.

0006-2960/85/0424-1817$01.50/0

that the activity of intermediates is small, if not zero (Jaenicke, 1982; Jaenicke & Rudolph, 1983), although there is evidence for at least partial activity in the cases of rabbit muscle aldolase (Chan et al., 1973; Rudolph et al., 1977) and rabbit muscle creatine kinase (Grossman et al., 1981). In the former case, the activity of the intermediates differs from that of the native enzyme in being sensitive to 2.3 M urea (Chan et al., 1973; Rudolph et al., 1977). In earlier work (Hermann et al., 1983), we have studied the reconstitution of the tetrameric enzyme phosphoglycerate mutase (EC 2.7.5.3) from yeast, after denaturation in 4 M guanidine hydrochloride (Gdn·HCl).[1] Over a range of concentrations, the kinetics of reassociation could be quantitatively described by a model of the type

$$4M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2D \xrightarrow{k_2} T$$

where M, D, and T represent monomer, dimer, and tetramer, respectively, and where the product of reconstitution, T, is indistinguishable from native enzyme. The values k_1, k_{-1}, and k_2 (in 50 mM sodium phosphate buffer, pH 7.5 at 20 °C) were 6.25×10^3 M^{-1} s^{-1}, 6.0×10^{-3} s^{-1}, and 2.75×10^4 M^{-1} s^{-1}, respectively. The regain of activity determined after incubation of samples with trypsin to eliminate refolding and reassociation during the assay [cf. Chan et al. (1973)] correlated with the appearance of tetramer. This indicated that neither monomeric nor dimeric intermediates possessed enzyme activity; however, they possessed considerable folded structure, as judged by the changes in protein fluorescence (Hermann et al., 1983). During some preliminary measurements, it was noted that omission of the trypsin treatment prior to the assay led to more rapid reactivation, suggesting that monomeric and dimeric intermediates may possess enzyme activity which is sensitive to trypsin. The present paper explores this possibility and presents evidence to show that the intermediates do indeed possess significant enzyme activity.

Materials and Methods

Phosphoglycerate mutase was isolated from bakers' yeast as described previously (Price & Jaenicke, 1982); the homogeneity of the preparation was shown by equilibrium ultracentrifugation and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Price & Jaenicke, 1982). The concentration of enzyme was determined spectrophotometrically by using the value $E^{0.1\%}_{280nm} = 1.45$ (Edelhoch et al., 1957); enzyme assays used the enolase coupled assay procedure (Grisolia, 1962).

Denaturation of the enzyme in 4 M Gdn·HCl and subsequent renaturation by 40-fold dilution, as well as cross-linking by glutaraldehyde and analysis of the data, were performed as described previously (Hermann et al., 1983).

Fluorescence measurements were made at 20 °C with a Hitachi Perkin-Elmer MPF 44A fluorescence spectrophotometer equipped with a corrected spectra accessory.

All chemicals, substrates, and enzymes were of analytical grade purity, and quartz-bidistilled water was used throughout.

Results

Renaturation in the Absence of Substrate. As reported previously, tetrameric yeast phosphoglycerate mutase (M_r 110000 ± 4000) does not show any concentration-dependent dissociation at $c > 5$ μg/mL (Price & Jaenicke, 1982); 4 M Gdn·HCl causes complete dissociation into subunits (M_r 28000 ± 2300); rapid dilution leads to high (>95%) recovery of native enzyme activity (Hermann et al., 1983). In an earlier work (Hermann et al., 1983), trypsin was added in order to avoid refolding and reassociation during the enzyme assays (Chan et al., 1973). In the present experiments, the effect of trypsin on the time course of reactivation was investigated.

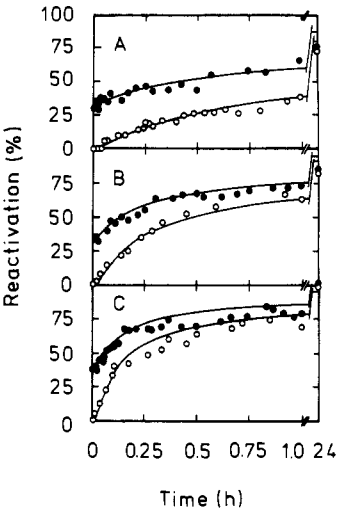

FIGURE 1: Kinetics of reactivation of yeast phosphoglycerate mutase in the absence of substrate. Yeast phosphoglycerate mutase was denatured in 4 M Gdn·HCl (15 s, 20 °C) and renatured by 1:40 dilution in 50 mM sodium phosphate buffer, pH 7.5, and 1 mM DTE, $T = 20$ °C. Tetramerization was determined by fixation of aliquots of reconstituting enzyme with 1% (w/v) glutaraldehyde (Hermann et al., 1983). Phosphoglycerate mutase concentration during reconstitution (μg/mg): 10.6 (A); 20.6 (B); 36.9 (C). (○) Kinetics of reactivation in the presence of trypsin. Aliquots of the reconstituting enzyme taken at times indicated were treated with 20 μg/mL trypsin (5 min, 20 °C) prior to the enzyme assay; full lines calculated for tetramer formation (cf. Hermann et al. (1983)]. (●) Kinetics of reactivation without trypsin treatment. Profiles calculated on the basis of the population analysis of association intermediates (Hermann et al., 1983), assuming monomers and dimers to exhibit 35% activity and tetramers 100%.

Figure 1 shows the results of experiments in which the regain of activity of yeast phosphoglycerate mutase during renaturation was studied. At each of the concentrations studied, there is a clear difference between the activity observed after 1–20-min incubation with trypsin (open symbols) and that observed without trypsin treatment (closed symbols).[2] The former data exactly match the regain of tetrameric structure as determined by the cross-linking technique. However, the latter data (obtained in the absence of trypsin) can only be accounted for by assuming that the intermediates (monomeric and dimeric) possess catalytic activity; as shown in Figure 1, there is an excellent fit to the data if it is assumed that the monomeric and dimeric intermediates each possess 35% of the specific activity of the native enzyme and that the tetrameric enzyme possesses 100% of the activity of native enzyme. Clearly the activity of the monomeric and dimeric intermediates, but not that of the tetrameric enzyme, is destroyed by incubation with trypsin under conditions under which the native enzyme is resistant to trypsin (Hermann et al., 1983).

Renaturation in the Presence of Substrate. In order to establish that the intermediates in the folding process do possess enzyme activity, it is necessary to exclude the possibility that there is rapid association (of monomers to dimers and/or

[1] Abbreviations: BPG, 2,3-bisphosphoglycerate; DTE, dithioerythritol; Gdn·HCl, guanidine hydrochloride; LDH, lactate dehydrogenase; 3-PGA, 3-phosphoglycerate; Tris, tris(hydroxymethyl)aminomethane; PGM, phosphoglycerate mutase.

[2] There was no effect of variation of the time of incubation with trypsin from 1 to 20 min. The standard incubation period was chosen as 5 min.

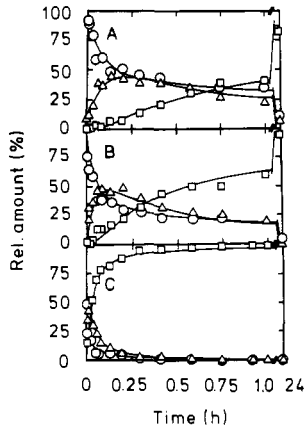

FIGURE 2: Kinetics of reassociation of yeast phosphoglycerate mutase in the presence of substrate. Experimental conditions as in Figure 1, except 12 mM 3-phosphoglycerate and 0.34 mM 2,3-bisphosphoglycerate were added to the renaturation buffer. Phosphoglycerate mutase concentration during reconstitution (μg/mL): 0.84 (A); 1.67 (B); 20.4 (C). Calculation of the curves as described by Hermann et al. (1983) with the following rate constants: $k_{1s} = 8.0 \times 10^4$ M^{-1} s^{-1}; $k_{-1s} = 8.5 \times 10^{-4}$ s^{-1}; $k_{2s} = 6.0 \times 10^4$ M^{-1} s^{-1}. (○) Monomers; (△) dimers; (□) tetramers.

dimers to tetramers) during the assay. Although the enhancing effects of coenzymes or substrates on the rate of reconstitution have been shown to be insignificant for a number of enzymes (Jaenicke & Rudolph, 1983), there are examples known where "nucleation" is of importance (Krebs et al., 1979; Jaenicke et al., 1980). In the present case, it is difficult to determine the effects of substrate directly, since the conditions for the cross-linking experiments and for the enzyme assays cannot easily be standardized. The enzyme activity is measured at 30 °C in the presence of 12 mM 3-PGA and 0.34 mM BPG in 30 mM Tris·HCl buffer, pH 7.0 [several polyvalent anions such as phosphate, sulfate, etc. are known to interfere with the activity of phosphoglycerate mutase (Ray & Peck, 1972)]. The renaturation and cross-linking experiments are performed at 20 °C in 50 mM sodium phosphate buffer, pH 7.5 (cross-linking with glutaraldehyde cannot be performed in the highly nucleophilic Tris buffer). However, the effect of substrates on the kinetics of regain of activity and regain of quaternary structure in phosphate buffer could be studied. Cross-linking experiments (Figure 2) show that there is indeed a substantial effect of substrates on the reassociation. Over the range of concentrations from 0.84 to 10.4 μg/mL, the reassociation data could again be fitted (see Figure 2) by the model

$$4M \underset{k_{-1s}}{\overset{k_{1s}}{\rightleftharpoons}} 2D \xrightarrow{k_{2s}} T$$

with the rate constants k_{1s}, k_{-1s}, and k_{2s} equal to 8.0×10^4 M^{-1} s^{-1}, 8.5×10^{-4} s^{-1}, and 6.0×10^4 M^{-1} s^{-1}. The presence of substrates has shifted the equilibrium between monomer and dimer to the side of the dimeric species (the association constant, obtained by dividing k_{1s} by $2k_{-1s}$ is approximately 90 times larger in the presence of substrate compared with its absence). The rate constant for association of the dimeric to the tetrameric species is only increased by a factor of approximately 2.

Although the rate constants of the association steps are increased in the presence of substrate, they are still far too low to enable significant association to occur during the time required for assay (<5 min). The concentration of enzyme subunits in the assay is 1.8 nM; at this concentration, the half-time of a second-order association process with a rate constant of $< 8 \times 10^4$ M^{-1} s^{-1} is 115 min; in order to reduce

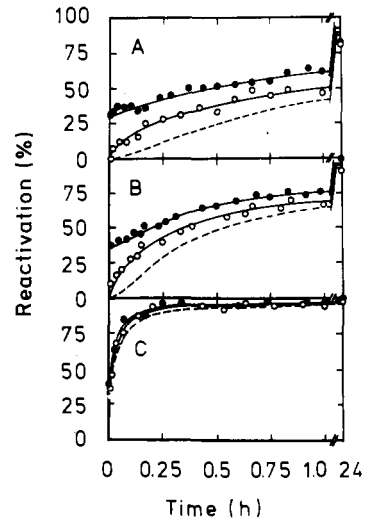

FIGURE 3: Kinetics of reactivation of yeast phosphoglycerate mutase in the presence of substrate. Experimental conditions as in Figure 2. Phosphoglycerate mutase concentration (μg/mL): 0.84 (A); 1.67 (B); 20.4 (C). (---) Kinetics of tetramerization (taken from Figure 2). (○) Residual enzymatic activity after trypsin treatment. Aliquots of the reconstituting enzyme, taken at the times indicated, were incubated with 20 μg/mL trypsin (5 min, 20 °C). Full lines calculated on the basis of the population analysis given in Figure 2, assuming monomers, dimers, and tetramers to exhibit 0%, 35%, and 100% specific activity, respectively. (●) Enzymatic activity without trypsin treatment. Full lines calculated by assuming monomers, dimers, and tetramers to exhibit 35%, 35%, and 100% activity, respectively.

the half-time to 5 min, the rate constant would have to be $\sim 2 \times 10^6$ M^{-1} s^{-1}.

The presence of substrates also has a substantial effect on the kinetics of reactivation (Figure 3, compare Figure 1). As shown in Figure 3, there is still a difference between the activity observed after a 5-min treatment with trypsin (open symbols) and that observed without trypsin treatment (closed symbols). Even after the trypsin treatment, the regain of activity runs ahead of the regain of quaternary structure (Figure 3). The data can be fitted by assuming that (in the absence of trypsin) the monomeric and dimeric intermediates each possess 35% of the activity of native enzyme and the tetrameric species 100% of the activity of native enzyme; in the presence of trypsin, the activity of the monomeric intermediate is zero, whereas those of the dimeric and tetrameric species remain at 35% and 100%, respectively, of native enzyme. Thus, the presence of substrate renders the dimeric but not the monomeric species resistant to trypsin.

As stated above, it is not possible to determine the kinetics of reassociation in this buffer by using the cross-linking technique. However, the kinetics of reactivation under the conditions used for assay (30 mM Tris·HCl, pH 7.0, 12 mM 3-PGA, and 0.34 mM BPG, 30 °C) can be compared with those obtained in 50 mM sodium phosphate buffer, pH 7.5, at 20 °C. The results obtained under the former conditions at an enzyme concentration of 0.84 μg/mL (Figure 4) are to be compared with those obtained under the latter (Figure 3A). The striking similarity between the results (obtained with or without trypsin treatment) indicates that there are no significant differences between the kinetics of reactivation in the two buffer systems and thus confirms the validity of the deductions drawn above about the activity and stability toward trypsin of monomeric and dimeric intermediates formed during the process of reconstitution.

Changes in Protein Fluorescence during Reconstitution in the Presence of Substrates. As shown in earlier work (Hermann et al., 1983), the changes in protein fluorescence during

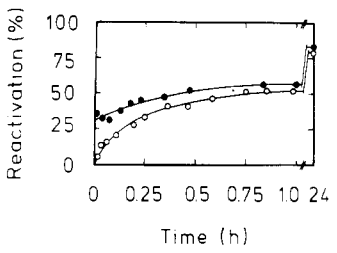

FIGURE 4: Reactivation of yeast phosphoglycerate mutase under the conditions of the enzymatic assay. Denaturation as in Figure 1. Phosphoglycerate mutase concentration 0.84 μg/mL. Reactivation by 1:40 dilution in 30 mM Tris·HCl, pH 7.0, 12 mM phosphoglycerate, and 0.34 mM 2,3-bisphosphoglycerate at 30 °C. (○) Enzymatic activity after trypsin treatment (cf. Figure 3). (●) Activity without trypsin treatment.

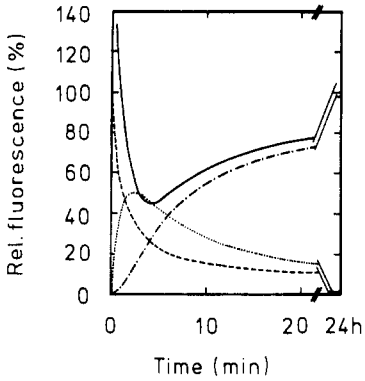

FIGURE 5: Kinetics of renaturation of yeast phosphoglycerate mutase in the presence of substrate. Changes in relative fluorescence at 328 nm (λ_{exc} = 279 nm). Denaturation–renaturation as in Figure 2. Phosphoglycerate mutase concentration 5.21 μg/mL. (—) Relative fluorescence (native enzyme taken as 100%); (- - -) time-dependent distribution of monomers; (···) time-dependent distribution of dimers; (–·–) time-dependent distribution of tetramers (cf. Figure 2).

reconstitution of yeast phosphoglycerate mutase in the absence of substrate could be accounted for by assuming that structured monomers formed in a fast folding step acquire 22% of the native enzyme fluorescence, while dimeric and tetrameric species each possess unchanged native enzyme fluorescence. In the presence of substrates, however, the changes are more complex. Figure 5 shows the data obtained at a protein concentration of 5.2 μg/mL in the presence of substrates (12 mM 3-PGA and 0.34 mM BPG) in 50 mM phosphate buffer, pH 7.5, at 20 °C. The time dependence of the relative concentrations of monomeric, dimeric, and tetrameric species (deduced from cross-linking) is also shown in Figure 5. The data suggest that there is a short-lived intermediate which shows a higher fluorescence than the native enzyme. Since in the absence of substrate this fluorescence "peak" is not observed, it would be reasonable to conclude that the short-lived intermediate is a monomeric, substrate-binding intermediate. Similar behavior, i.e., multiphasic changes in protein fluorescence during reconstitution, has been observed in a number of other systems, e.g., the β_2 dimer of tryptophan synthase from *Escherichia coli* (Seifert et al., 1985), rabbit muscle aldolase (Rudolph et al., 1976), and LDH–H_4 from pig heart (Jaenicke & Rudolph, 1977), and points to the occurrence of multiple changes in the environments of particular fluorophores during the folding process.

Discussion

The results of studies of the reconstitution of a number of oligomeric enzymes have shown that the native quaternary structure is a prerequisite for the expression of catalytic activity. Examples include alcohol dehydrogenase, malate dehydrogenase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, the β_2 dimer of *E. coli* tryptophan synthase, and fumarase (Yamoto & Machuri, 1979; Jaenicke, 1984). However, in the cases of creatine kinase (Grossman et al., 1981) and aldolase (Chan et al., 1973; Rudolph et al., 1977), there is strong kinetic evidence for at least partial activity of intermediate species during reconstitution. In this paper, we have presented evidence to show that the monomeric and dimeric intermediates formed during the reconstitution of yeast phosphoglycerate mutase possess significant activity; this activity is sensitive to trypsin in the absence of substrates (3-PGA and BPG), but in the presence of substrates, the activity of the dimeric intermediate is stable toward trypsin. The results from X-ray crystallography (Winn et al., 1981) show that the four active sites in the native enzyme are well separated from one another, and the observation of activity in the monomeric species is consistent with the finding that this species has considerable secondary structure as judged by circular dichroism (Hermann et al., 1983). However, the sensitivity of these intermediates toward trypsin compared with the very slight sensitivity of the native enzyme (Hermann et al., 1983) points to a more compact structure for the latter.

There are a number of other examples of tetrameric enzymes in which the sensitivity of dimeric intermediates to proteolysis is much greater than that of the native enzyme. In their studies on the reconstitution of pig heart fumarase, Yamoto & Murachi (1979) found that an inactive dimeric species could be obtained following removal of the denaturant (urea) by dialysis. This dimer possessed similar structure to the native enzyme as judged by fluorescence and circular dichroism but was very sensitive to trypsin whereas the native enzyme was resistant. Schultze & Colowick (1969) showed that the tetrameric form of yeast hexokinase was stable toward trypsin, whereas the dimeric form produced by the addition of glucose or salts was highly susceptible to trypsin.

Recent work in this laboratory (Girg et al., 1983a) has shown that a dimeric intermediate on the pathway of reconstitution of pig skeletal muscle lactate dehydrogenase can be trapped by limited proteolysis with thermolysin. This intermediate in which the chains lack an N-terminal segment which is essential in stabilizing the native, tetrameric structure (Holbrook et al., 1975) is much more susceptible to proteolysis than is the native enzyme. The LDH dimer is inactive under normal assay conditions, but in the presence of "structure-making" ions such as 2 M ammonium sulfate, it shows about 40% of the activity of the native enzyme. It has been subsequently shown that the unmodified dimeric intermediate shows approximately 50% of the activity of native enzyme in the presence of 2 M ammonium sulfate (Girg et al., 1983b).

It would appear that in all these cases the native quaternary structure is more compact or less flexible than that of the dimeric intermediate, thereby providing protection against proteolysis (Jaenicke, 1984).

In conclusion, the results described in this paper show that monomeric and dimeric intermediates produced during the reconstitution of yeast phosphoglycerate mutase possess significant activity. The results also indicate that the use of trypsin to prevent refolding and reassociation of enzymes during assays (Chan et al., 1973) can prevent the observation of active, trypsin-sensitive intermediates, and thus conclusions regarding the activity of such intermediates need to be drawn with considerable care.

Registry No. Gdn·HCl, 50-01-1; BPG, 138-81-8; phosphoglycerate mutase, 9032-62-6.

REFERENCES
Chan, W. W.-C., Mort, J. S., Chong, D. K. K., & Macdonald, P. D. M. (1973) *J. Biol. Chem. 248*, 2778–2784.
Edelhoch, H., Rodwell, V. W., & Grisolia, S. (1957) *J. Biol. Chem. 228*, 891–903.
Girg, R., Jaenicke, R., & Rudolph, R. (1983a) *Biochem. Int. 7*, 433–441.
Girg, R., Rudolph, R., & Jaenicke, R. (1983b) *FEBS Lett. 163*, 132–135.
Grisolia, S. (1962) *Methods Enzymol. 5*, 236–242.
Grossman, S. H., Pyle, J., & Steiner, R. J. (1981) *Biochemistry 20*, 6122–6128.
Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry 20*, 5195–5201.
Hermann, R., Rudolph, R., Jaenicke, R., Price, N. C., & Scobbie, A. (1983) *J. Biol. Chem. 258*, 11014–11019.
Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossmann, M. G. (1975) *Enzymes, 3rd Ed. 11*, 191–292.
Jaenicke, R. (1982) *Biophys. Struct. Mech. 8*, 231–256.
Jaenicke, R. (1984) *Angew. Chem., Int. Ed. Engl. 23*, 395–413.
Jaenicke, R., & Rudolph, R. (1977) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) pp 351–367, de Gruyter, Berlin and New York.
Jaenicke, R., & Rudolph, R. (1983) in *Biological Oxidations* (Sund, H., & Ullrich, V., Eds.) pp 62–90, Springer-Verlag, Berlin and Heidelberg.
Jaenicke, R., Krebs, H., Rudolph, R., & Woenckhaus, C. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1966–1969.
Krebs, H., Rudolph, R., & Jaenicke, R. (1979) *Eur. J. Biochem. 100*, 359–364.
Price, N. C., & Jaenicke, R. (1982) *FEBS Lett. 143*, 283–286.
Ray, W. J., Jr., & Peck, E. T., Jr. (1972) *Enzymes, 3rd Ed. 6*, 407–477.
Rudolph, R., Engelhard, M., & Jaenicke, R. (1976) *Eur. J. Biochem. 67*, 455–462.
Rudolph, R., Westhof, E., & Jaenicke, R. (1977) *FEBS Lett. 73*, 204–206.
Schultze, I. T., & Colowick, S. P. (1969) *J. Biol. Chem. 244*, 2306–2316.
Seifert, T., Bartholmes, P., & Jaenicke, R. (1985) *Biochemistry 24*, 339–345.
Winn, S. I., Watson, H. C., Harkins, R. N., & Fothergill, L. A. (1981) *Philos. Trans. R. Soc. London, Ser. B 293*, 121–130.
Yamoto, S., & Murachi, T. (1979) *Eur. J. Biochem. 93*, 189–195.

Human Brain Monoamine Oxidase Type B: Mechanism of Deamination As Probed by Steady-State Methods†

L. Bruce Pearce and Jerome A. Roth*
Department of Pharmacology and Therapeutics, State University of New York at Buffalo, School of Medicine, Buffalo, New York 14214
Received August 1, 1984

ABSTRACT: Recently, evidence has been published which suggests that [Husain, M., Edmondson, D. E., & Singer, T. P. (1982) *Biochemistry 21*, 595–600] monoamine oxidase [amine:oxygen oxidoreductase (MAO), EC 1.4.3.4] deaminates phenylethylamine and benzylamine via two distinct kinetic pathways which involve either binary or ternary complex formation, respectively. These conclusions were drawn largely from stopped-flow kinetic analysis performed on purified enzyme removed from its native membrane and in the presence of the inhibitory detergent Triton X-100. In this study, *d*-amphetamine and alternative substrates were used as steady-state probes of the kinetics of deamination by the B form of human brain MAO using native membrane-bound enzyme. Initial velocity studies showed mixed-type patterns for amphetamine inhibition of phenylethylamine, tryptamine, and tyramine when either amine or oxygen was the varied substrate. Slope and intercept vs. amphetamine concentration replots were linear in all cases except for phenylethylamine (hyperbolic); K_i values obtained from linear replots of slope or intercept values were comparable. In contrast, amphetamine was a competitive inhibitor of benzylamine deamination when amine concentration was varied and uncompetitive when oxygen concentration was varied; slope and intercept replots were linear for both. When benzylamine was the alternative substrate inhibitor and tyramine and tryptamine deamination was measured, mixed-type inhibition patterns were obtained when either amine or oxygen concentration was varied; replots of slope and intercept were linear in all cases. These results strongly support the proposal that phenylethylamine and benzylamine are deaminated via two distinct mechanisms and further suggest that tyramine and tryptamine, like phenylethylamine, are deaminated via exclusively binary complex formation whereas benzylamine deamination uniquely involves the formation of a ternary complex with the reduced form of the oxidase.

Numerous studies have suggested that deamination of amines by the membrane-bound outer mitochondrial enzyme monoamine oxidase [amine:oxygen oxidoreductase (MAO), EC 1.4.3.4] occurs via a ping-pong mechanism (Tipton, 1968; Oi et al., 1970, 1971; Houslay & Tipton, 1973; Fowler & Oreland, 1979; Roth, 1979). However, Husain et al. (1982) have recently proposed that the mechanism of deamination of monoamines by bovine liver type B MAO may be substrate dependent, and they suggest that deamination of benzylamine and phenylethylamine proceeds via two distinct pathways. According to their model, deamination may involve either

† This study was supported by a grant from the National Institutes of Health (GM 7145-10).

0006-2960/85/0424-1821$01.50/0