were found among the binding sites on MP-2 and MP-3, it is not clear at present whether such differences are attributable to multiple independent sites on one or more membrane proteins or to cooperative interactions between sites on either the same membrane protein or the predominant protein and minor contaminants. The apparent increases in the net binding activity of receptors for formylmethionyl peptides on intact neutrophils treated with membrane perturbants (Liao & Freer, 1980) or on monocytes exposed to immunological stimuli (Pike et al., 1980) may be explained by the exposure of latent high-affinity receptors. The solubilization and purification of constituents of leukocyte chemotactic factor receptors that retain specific binding activity for the homologous stimulus may facilitate an understanding of the coupling of such receptors to a variety of distinct leukocyte functions.

References

Aswanikumar, S., Corcoran, B. A., Schiffmann, E., Day, A. R., Freer, R. J., Showell, H. J., Becker, E. L., & Pert, C. B. (1977) *Biochem. Biophys. Res. Commun. 74*, 810–817.

Boyum, A. (1968) *Scand. J. Clin. Lab. Invest. Suppl. 21*, (*No. 97*), 31–48.

Bretz, U., & Baggiolini, M. (1974) *J. Cell Biol. 63*, 251–296.

Dayhoff, M. O., Hunt, L. T., & Hurst-Caldrone, S. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., Ed.) Vol. 5, pp 363–375, National Biomedical Research Foundation, Washington, DC.

Furthmay, H., Tomita, M., & Marchesi, V. T. (1975) *Biochem. Biophys. Res. Commun. 65*, 113–121.

Goetzl, E. J., & Austen, K. F. (1972) *J. Exp. Med. 136*, 1564–1580.

Goetzl, E. J., & Hoe, K. Y. (1979) *Immunology 37*, 407–418.

Goetzl, E. J., & Pickett, W. C. (1980) *J. Immunol. 125*, 1789–1791.

Goetzl, E. J., & Pickett, W. C. (1981) *J. Exp. Med. 153*, 482–487.

Goldman, D. W., Pober, J. S., White, J., & Bayley, H. (1979) *Nature (London) 280*, 841–843.

Hare, P. E. (1975) *Mol. Biol., Biochem. Biophys. 8*, 201–210.

Ho, M. K., & Guidotti, G. (1975) *J. Biol. Chem. 250*, 75–76.

Klempner, M. S., Mikkelsen, R. B., Corfman, D. H., & Andre-Schwartz, J. (1980) *J. Cell Biol. 86*, 21–28.

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

Liao, C. S., & Freer, R. J. (1980) *Biochem. Biophys. Res. Commun. 93*, 566–571.

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

Niedel, J. E., Kahane, I., & Cuatrecases, P. (1979) *Science (Washington, D.C.) 205*, 1412–1414.

Niedel, J., Davis, J., & Cuatrecasas, P. (1980) *J. Biol. Chem. 255*, 7063–7066.

O'Flaherty, J. T., Showell, H. J., Kreutzer, D. L., Ward, P. A., & Becker, E. L. (1978) *J. Immunol. 120*, 1326–1340.

Pike, M. C., Fischer, D. G., Koren, H. S., & Snyderman, R. (1980) *J. Exp. Med. 152*, 31–40.

Showell, H. J., Freer, R. J., Zigmond, S. H., Schiffmann, E., Aswanikumar, S., Corcoran, B. A., & Becker, E. L. (1976) *J. Exp. Med. 143*, 1154–1169.

Stewart, P. S., MacLennan, D. M., & Shamoo, A. (1976) *J. Biol. Chem. 251*, 712–719.

Williams, L. T., Snyderman, R., Pike, M. C., & Lefkowitz, R. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1204–1208.

Roles of Arginyl Residues in Pyridoxamine-5′-phosphate Oxidase from Rabbit Liver†

Jung-Do Choi and Donald B. McCormick*

ABSTRACT: Pyridoxamine-5′-phosphate oxidase (pyridoxine-5′-phosphate oxidase) is inactivated by arginine-specific reagents. Inactivation by phenylglyoxal follows pseudo-first-order kinetics and is first order with respect to modifier. The substrate-competitive inhibitors pyridoxal 5′-phosphate oxime and 4′-deoxypyridoxime 5′-phosphate and product pyridoxal 5′-phosphate protect holoenzyme against inactivation but have no significant effect on the inactivation of apoenzyme. The extent of protection is dependent on their respective binding constants. Extrapolation to complete inactivation shows modification of ~4 out of the 40 total arginyl residues in the native enzyme, with ~1 residue protected by pyridoxal 5′-phosphate, as determined by incorporation of [7-^{14}C]-phenylglyoxal. Binding of coenzyme flavin mononucleotide increases the rate of inactivation 3-fold by enhancing reactivity of an essential arginyl residue toward reagent. This and the fact that substrates and product do not bind well to apoenzyme indicate that an arginyl residue essential for substrate/product binding is exposed by formation of functional holoenzyme. The oxidase is also inactivated by either 2,3-butanedione in borate buffer or 2,4-pentanedione in phosphate buffer and is similarly protected by pyridoxal 5′-phosphate. Inactivation by butanedione is fully reversible on removal of excess butanedione and borate. Inability of hydroxylamine to restore activity of pentanedione-modified oxidase suggests that inactivation is due to modification of arginyl, not lysyl, residues. Unlike native enzyme, modified enzyme is not able to bind product pyridoxal 5′-phosphate. These results provide evidence for the essential role of an arginyl residue in the binding of substrate/product to the enzyme and suggest the possible shielding of many arginyls in ionic association of the two polypeptide chains of the monomer.

Pyridoxamine-5′-phosphate oxidase (pyridoxine-5′phosphate oxidase) (EC 1.4.3.5) catalyzes the conversion of the phosphorylated B_6 vitamers to coenzyme pyridoxal 5′-phosphate (McCormick & Merrill, 1980). It has been postulated to be one of the regulating steps in vitamin B_6 metabolism, since product pyridoxal 5′-phosphate is strongly inhibitory (Snell & Haskell, 1971; Merrill et al., 1978). The oxidase from rabbit liver contains one FMN per 54 000 molecular weight

† From the Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322. *Received April 2, 1981*. This investigation was supported by a grant from the National Institutes of Health (AM 26746).

0006-2960/81/0420-5722$01.25/0

monomer and is composed of two noncovalently linked subunits with blocked N termini (Kazarinoff & McCormick, 1975; McCormick et al., 1976). Our studies with numerous analogues of flavin mononucleotide (FMN) (Tsibris et al., 1966; Kazarinoff & McCormick, 1974, 1975; Merrill et al., 1979a) and pyridoxyl 5′-phosphate (Kazarinoff & McCormick, 1974; Merrill et al., 1980) have provided some information concerning the properties of coenzyme and substrate/product binding sites in the oxidase. Continuing studies have been aimed at elucidating the nature and role of amino acid residues within the active site of the oxidase. The amino acid composition (Tsuge & McCormick, 1980) and nature of a number of crucial amino acid residues have been determined. Chemical modifications have shown that one from among six sulfhydryl is critical for catalytic function (McCormick et al., 1976; Tsuge & McCormick, 1980), at least one of six trytophanyl residues is involved in the binding of coenzyme (McCormick, 1977), and one histidyl residue is essential for substrate binding and probably functions as base for proton abstraction (Horiike et al., 1979b). In a large number of enzymes, arginyl residues play a general role as anion recognition sites for negatively charged substrates and cofactors [Riordan et al., (1977) and references cited therein]. Since both substrate (Korytnyk et al., 1972; Kazarinoff & McCormick, 1973) and coenzyme (Wada & Snell, 1961; Kazarinoff & McCormick, 1974) anionic phosphate groups are required, it seemed likely that positively charged arginyl residues would be involved in the binding of these ligands to the oxidase. The present paper describes studies on the chemical modification of arginyl residues in pyridoxamine-5′-phosphate oxidase with three well-characterized reagents, viz., phenylglyoxal (Takahashi, 1968), 2,3-butanedione (Riordan, 1973), and 2,4-pentanedione (Gilbert & O'Leary, 1975). The results described herein demonstrate that the oxidase is inactivated by modification of an arginyl residue essential for the binding of substrate/product to the enzyme.

Experimental Procedures

Materials. Pyridoxamine-5′-phosphate oxidase was purified from rabbit liver as described previously [Kazarinoff & McCormick (1975), with minor modifications by Merrill et al. (1979b)]. Phenylglyoxal monohydrate, 2,3-butanedione, and 2,4-pentanedione were from Aldrich Chemical Co. [7-^{14}C]Phenylglyoxal, prepared from [7-^{14}C]acetophenone (ICN) by selenium oxidation (Riley & Gray, 1943), had a specific activity of 0.35 mCi/mmol. Pyridoxamine 5′-phosphate, pyridoxal 5′-phosphate, and FMN were purchased from Sigma Chemical Co. The commercial FMN was purified chromatographically (Moffatt & Khorana, 1958). *N*-(5′-Phospho-4′-pyridoxyl)-*N*′-(1-naphthyl)ethylenediamine (DePecol & McCormick, 1980), pyridoxal 5′-phosphate oxime (Heyl et al., 1951), 4′-deoxypyridoxine 5′-phosphate (Korytnyk & Ikawa, 1970), and pyridoxamine 5′-sulfate (Yang et al., 1974) were synthesized by published procedures. All other chemicals were of the highest commercial grade.

Assay. Oxidase activity was measured by spectrophotometric quantitation of the phenylhydrazone of pyridoxal 5′-phosphate (ϵ_{410} = 23 000 M^{-1} cm^{-1}) using the method of Wada & Snell (1961). For the enzyme samples incubated with phenylglyoxal or butanedione, the fluorometric assay was employed (DePecol & McCormick, 1980). Enzymatic activity was assayed at 25 °C by continuous monitoring of the increase of fluorescence at 430 nm upon conversion of substrate to products. A standard assay mixture contains 20–80 μM *N*-(5′-phospho-4′-pyridoxyl)-*N*′-(1-naphthyl)ethylenediamine as substrate, 0.5 μM FMN, and bovine serum albumin (10 μg/mL) in 0.2 M Tris-HCl buffer (pH 8.0). Protein concentrations were determined by either the biuret method (Gornall et al., 1949) or that of Lowry et al. (1951), with bovine serum albumin as the standard. A molecular weight of 54 000 (Kazarinoff & McCormick, 1975) was used to determine the molar concentration of the oxidase.

Measurements of Absorbance and Fluorescence. Routine absorption measurements were made by using a Beckman 24 spectrophotometer. Absorption spectra and spectrophotometric titration of native and modified enzyme with pyridoxal 5′-phosphate were obtained with a Cary 219 spectrophotometer. Fluorescence measurements were made with a Perkin-Elmer MPF 44B spectrofluorometer equipped with a DCSU-2 differential corrected spectra unit.

Modification with Phenylglyoxal. Modification reactions with phenylglyoxal were carried out at 25 °C in 0.1 M potassium phosphate buffer (pH 8.0) containing 5% (v/v) ethanol, unless otherwise indicated. All other conditions was indicated in the figure and table legends. Modification reactions were initiated by adding reagent to the enzyme solution which had been preincubated for 10 min at 25 °C. Aliquots of reaction mixture were removed at time intervals and assayed for activity. Modification was found not to proceed further during the activity assay. Activity is expressed as the ratio of the activity of the modified enzyme, V, to that of the control subjected to the same conditions but in the absence of modifying reagent, V_c, multiplied by 100. The number of arginyl residues modified was determined by incorporation of [^{14}C]-phenylglyoxal. After the enzyme solution was incubated with [^{14}C]phenylglyoxal, excess radioactive phenylglyoxal was separated by gel filtration through a Sephadex G-25 column (0.9 × 15 cm) with 10 mM potassium phosphate (pH 8.0) as eluant. Following gel filtration, the enzyme solution was immediately assayed for enzymatic activity, and the protein concentration was determined by the method of Lowry et al. (1951). The amount of [^{14}C]phenylglyoxal incorporated was determined by using an aqueous counting scintillant (ACS Amersham) with a Beckman LS 3133T scintillation counter. The number of arginyl residues modified was calculated with the stoichiometry of 2 mol of phenylglyoxal/mol of arginine (Takahashi, 1968).

Amino Acid Analyses. Amino acid analyses were used to quantitate the arginyl residues modified by phenylglyoxal. The enzyme was reacted with phenylglyoxal in 0.1 M potassium phosphate buffer (pH 8.0) at 25 °C. Samples were removed at time intervals and the reactions stopped by gel filtration on Sephadex G-25 with 10 mM potassium phosphate (pH 8.0) as eluant. These samples were acidified with 5 N acetic acid (final concentration of 0.5 N) to prevent regeneration of arginyl residues and then prepared for amino acid analyses. The samples were immediately dried under vacuum and hydrolyzed in evacuated, sealed ampules with 6 N HCl at 110 °C for 22 h. Amino acid analyses were performed on a Beckman 120B amino acid analyzer.

Modifications with 2,3-Butanedione and 2,4-Pentanedione. Modifications with butanedione and pentanedione were carried out as described in the procedure for modification with phenylglyoxal, but in a different buffer. The reversibility of butanedione inactivation upon removal of borate was determined by first inactivating (to 6% of control) the enzyme with butanedione in 50 mM borate (pH 8.0). The inactivated enzyme was then freed from borate by gel filtration over a Sephadex G-25 column with 0.1 M potassium phosphate (pH 8.0) as eluant. The modified enzyme solution obtained from elution was periodically assayed for activity over a period of

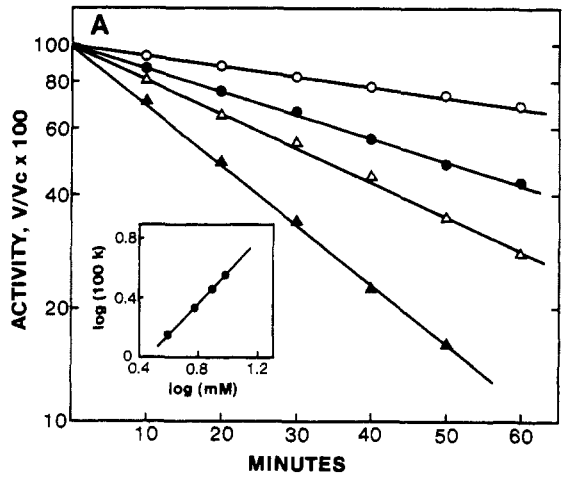

FIGURE 1: Inactivation of pyridoxamine-5′-phosphate oxidase by phenylglyoxal. Oxidase (2.1 μM) was incubated with 2 mM (○), 4 mM (●), 6 mM (△), and 9.8 mM (▲) phenylglyoxal as described under Experimental Procedures. (Insert) Plot of log (pseudo-first-order rate constant) vs. log [phenylglyoxal]. The native enzyme of the control retains full activity over this period of time.

incubation at 25 °C and compared with that of a control. The pentanedione-modified enzyme was tested for reactivation by treatment with hydroxylamine. The oxidase was inactivated by pentanedione to 10% residual activity and then separated from excess reagent by filtration on a Sephadex G-25 column. During incubation of the enzyme solution with 1 M hydroxylamine at 25 °C over a period of 4 h, enzymatic activity was periodically measured after filtration through a Sephadex G-25 column. The same manipulation of native enzyme without pentanedione treatment was done as a control.

Results

Inactivation by Phenylglyoxal. Pyridoxamine-5′-phosphate oxidase is inactivated by treatment with phenylglyoxal in the absence and presence of coenzyme FMN. Complete inactivation can be achieved by prolonged incubation with the reagent. Over the time necessary to completely inactivate the enzyme, the control enzyme sample retains full activity. As shown in Figure 1, inactivation follows pseudo-first-order kinetics, the rate of which is a function of reagent concentration. The second-order rate constants for the inactivation of apo- and holoenzyme (not shown) with phenylglyoxal in 0.1 M phosphate buffer at pH 8.0 are 3.7 M^{-1} min^{-1} and 11.1 M^{-1} min^{-1} at 25 °C, respectively. The reaction order with respect to phenylglyoxal was determined from the slope of a plot of log (pseudo-first-order rate constant) vs. log [phenylglyoxal] to be 1.10 and 1.05 for apo- and holoenzyme, respectively. Addition of FMN enhances the rate of inactivation 3-fold. An apparent dissociation constant (K_D) for FMN binding to both the native and inactivated enzyme with 10% residual activity was determined by fluorometric titration (Merrill et al., 1979a,b). Values of K_D of 12 nM for the native enzyme and 25 nM for modified enzyme were obtained. The value for native enzyme is in good agreement with that reported (Kazarinoff & McCormick, 1975; Merrill et al., 1979a,b).

Inactivation by 2,3-Butanedione. Another arginine reagent, 2,3-butanedione, also rapidly inactivates the oxidase in borate buffer (Figure 2). The inactivation follows pseudo-first-order kinetics and is dependent on butanedione concentration. However, the inactivation by butanedione in phosphate buffer is much slower than in borate buffer and reaches an equilibrium state. The inactivation of the oxidase by butanedione in borate buffer is fully reversible upon removal of borate

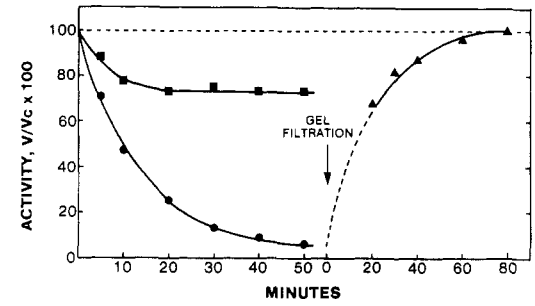

FIGURE 2: Inactivation of pyridoxamine-5′-phosphate oxidase by butanedione. The oxidase (2.1 μM) was incubated at 25 °C with 10 mM butanedione in the presence of 5 μM FMN in either 50 mM potassium borate, pH 8.0 (●), or 50 mM potassium phosphate, pH 8.0 (■). The enzyme inactivated in borate buffer was separated from excess butanedione and borate on a Sephadex G-25 column and periodically assayed for activity during incubation at 25 °C (▲). The arrow indicates the time at which sample was applied to the column.

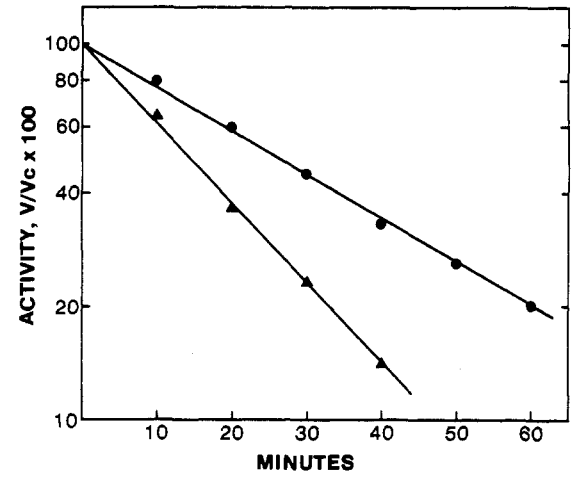

FIGURE 3: Inactivation of pyridoxamine-5′-phosphate oxidase by pentanedione. The oxidase (2.1 μM) was incubated with 0.3 M pentanedione in the absence (●) and presence (▲) of 5 μM FMN in 0.2 potassium phosphate (pH 8.0) at 25 °C.

(Figure 2). The oxidase, which lost 94% of its original activity by incubation with butanedione–borate, is restored to the same activity as the control within 80 min of incubation at 25 °C following removal of excess butanedione and borate. The characteristic of butanedione modification seen here has been observed for many other arginyl enzymes (Riordan, 1973; Daemen & Riordan, 1974; Foster & Harrison, 1974; Lange et al., 1974; Rohrbach & Bodley, 1977).

Inactivation by 2,4-Pentanedione. The time course for loss of activity upon incubation of apo- and holoenzyme with pentanedione is shown in Figure 3. Inactivation of both apo- and holoenzyme is first order in both reagent and the oxidase. As seen with phenylglyoxal, the rate of inactivation for the holoenzyme is greater than that for apoenzyme. The enzymatic activity of pentanedione-modified enzyme is not restored by treatment with 1 M hydroxylamine for 4 h at 25 °C, suggesting that modification of lysyl residues is not responsible for the inactivation.

Protection by Substrate-Competitive Inhibitors against Inactivation. The substrate-competitive inhibitors pyridoxal 5′-phosphate oxime and 4′-deoxypyridoxine 5′-phosphate and product pyridoxal 5′-phosphate protect the holoenzyme against inactivation by phenylglyoxal (Figure 4). However, the inhibitors have no significant protective effect on the inactivation of apoenzyme; this is consistent with the fact that they do not bind well to apoenzyme (Merrill et al., 1980). Pyridoxamine 5′-sulfate, which is not inhibitory for the oxidase (Kazarinoff

Table I: Protection by Pyridoxal 5'-Phosphate against Inactivation[a]

time (min)	activity [$(V/V_c) \times 100$]			
	BD[b]	BD + PLP[c]	PD[d]	PD + PLP
10	47	98	64	99
20	25	97	36	97
40	9	94	14	95

[a] Holoenzyme (2.1 μM) was incubated with either 10 mM butanedione in 50 mM potassium borate (pH 8.0) or 0.3 M pentanedione in 0.2 M potassium phosphate (pH 8.0) in the absence and presence of 30 μM pyridoxal 5'-phosphate as described under Experimental Procedures. [b] Butanedione. [c] Pyridoxal 5'-phosphate. [d] Pentanedione.

& McCormick, 1975), shows no such protective effect. The greater protection by pyridoxal 5'-phosphate and its oxime compared to 4'-deoxypyridoxine 5'-phosphate expectedly relates to their dissociation constants (K_D) which are 1.3 μM for both pyridoxal 5'-phosphate and its oxime and 3.7 μM for 4'-deoxypyridoxine 5'-phosphate (Merrill et al., 1980). The K_D for pyridoxal 5'-phosphate binding to holoenzyme was determined from rate constants for inactivation by phenylglyoxal in the presence of various concentration of pyridoxal 5'-phosphate by using the method of Horiike and McCormick (1980). The K_D of 0.86 μM obtained is in reasonable agreement with the reported value of 1.3 μM determined by direct spectrophotometric titration (Merrill et al., 1980). Pyridoxal 5'-phosphate also afforded similar protection against inactivation by butanedione and pentanedione (Table I). Addition of pyridoxal 5'-phosphate to holoenzyme which had been completely inactivated by phenylglyoxal did not cause changes in absorbance characteristic for the complex between the oxidase and pyridoxal 5'-phosphate (Merrill et al., 1980), indicating that inactivated oxidase is not able to bind product pyridoxal 5'-phosphate.

[7-^{14}C]Phenylglyoxal Incorporation. The number of arginyl residues modified by phenylglyoxal was determined by incorporation of [^{14}C]phenylglyoxal into the oxidase. The progressive incorporation of [^{14}C]phenylglyoxal accompanying the time-dependent inactivation of holoenzyme is shown in Figure 5. Only ~6 of the 40 arginyl residues in the native monomer are modified upon complete inactivation. Although inactivation of the oxidase shows simple first-order decay (Figure 1), the incorporation of phenylglyoxal is a complex function. Extrapolation of the linear portion yields 3.8 arginyl residues modified per monomer. In a parallel experiment, the oxidase was modified with [^{14}C]phenylglyoxal in the presence of pyridoxal 5'-phosphate. Pyridoxal 5'-phosphate prevents inactivation of holoenzyme and simultaneously protects arginyl residues from modification. The extent of inactivation and the number of arginyl residues modified by phenylglyoxal in the absence and presence of pyridoxal 5'-phosphate are listed

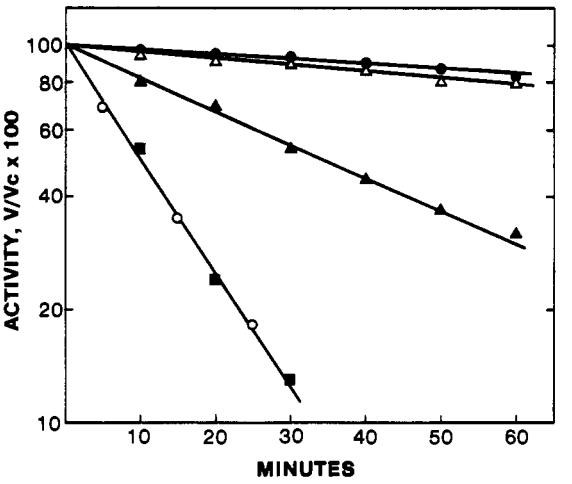

FIGURE 4: Protective effect of competitive inhibitors on the inactivation of the holoenzyme by phenylglyoxal. The oxidase (2.1 μM) plus 5 μM FMN was incubated with 6 mM phenylglyoxal in the presence of no inhibitors (○), 10 μM pyridoxal 5'-phosphate (●), 10 μM pyridoxal 5'-phosphate oxime (△), 10 μM 4'-deoxypyridox ine 5'-phosphate (▲), and 20 μM pyridoxamine 5'-sulfate (■) as described under Experimental Procedures.

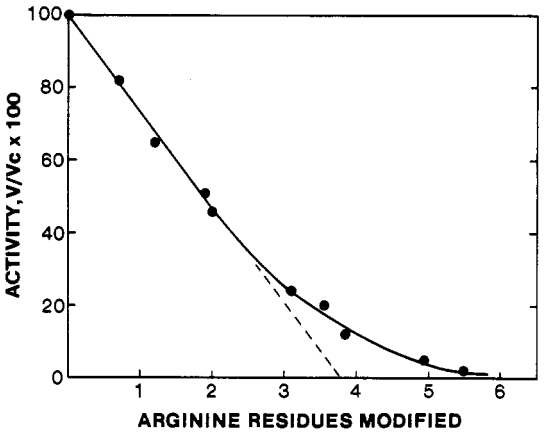

FIGURE 5: Correlation of inactivation of the oxidase with the number of arginyl residues modified. The number of arginyl residues modified was determined by incorporation of [7-^{14}C]phenylglyoxal and is expressed per enzyme monomer. The oxidase (3 μM) was incubated with 4 mM [7-^{14}C]phenylglyoxal in the presence of 7 μM FMN. Enzymatic activity and phenylglyoxal incorporation were determined as described under Experimental Procedures. The number of arginyl residues modified was calculated by using the stoichiometry of 2 mol of phenylglyoxal per mol of arginine (Takahashi, 1968).

Table II: Protection by Pyridoxal 5'-Phosphate against Incorporation of [7-^{14}C]Phenylglyoxal[a]

time (min)	inact. (%)	addition to incubation mixture: (1) none — no. of Arg modified	(2) pyridoxal 5'-phosphate — inact. (%)	(2) pyridoxal 5'-phosphate — no. of Arg modified	difference, (1) – (2) — inact. (%)	difference, (1) – (2) — no. of Arg modified	no. of Arg modified for 100% inact.[b]
4	18	0.69	1	0.49	17	0.20	1.18
15	49	1.89	3	1.25	46	0.64	1.39
30	76	3.12	5	2.26	71	0.86	1.21
45	88	3.86	6	2.92	82	0.94	1.15
60	95	4.96	7	3.94	88	1.02	1.14

[a] The apoenzyme (3 μM) plus FMN (7 μM) was incubated with 4 mM [7-^{14}C]phenylglyoxal in 0.1 M phosphate buffer (pH 8.0) at 25 °C for the time periods indicated in the absence and presence of 30 μM pyridoxal 5'-phosphate. Activity and [7-^{14}C]phenylglyoxal incorporation were determined as described under Experimental Procedures. The number of arginyl residues modified was calculated by using the stoichiometry of 2 mol of phenylglyoxal/mol of arginine (Takahashi, 1968). [b] Calculated from [100%/(difference in % inactivation)] × difference in number of arginines modified. The native enzyme of the control retains full activity over this time period.

Table III: Effect of Coenzyme and Inhibitor on Modification Followed by Amino Acid Analyses[a]

	none		FMN		FMN + PLP[b]		PLP	
time (min)	inact. (%)	loss of Arg	inact. (%)	loss of Arg	inact. (%)	loss of Arg	act. (%)	Arg
15	22	2.5	47	2.2	2	1.8	45	0.4
60	60	5.2	94	5.4	6	4.2	88	1.2

[a] The oxidase (3 μM) was incubated with 4 mM phenylglyoxal for the time periods indicated in the absence and presence of 7 μM FMN and 30 μM pyridoxal 5′-phosphate. Activity measurements and amino acid analyses were carried out as described under Experimental Procedures. [b] Pyridoxal 5′-phosphate. All results are averages from two or more analyses. The native enzyme of the control retains full activity over this period of time.

in Table II. The number of arginyl residues that are essential for activity and protected by pyridoxal 5′-phosphate was obtained by comparison at the same incubation time to the total number of arginyl residues modified and the extent of inactivation for samples incubated in the absence and presence of pyridoxal 5′-phosphate. If the difference in modified arginyl residues is linearly related to the difference in inactivation, pyridoxal 5′-phosphate protects 1.2 arginyl residues, and modification of this arginyl residue causes inactivation of the oxidase.

Amino Acid Analyses. Amino acid analyses of native and phenylglyoxal-modified oxidase reveal that arginine is the only amino acid detectably modified by treatment with phenylglyoxal. The extent of inactivation and number of arginyl residues modified by treatment with phenylglyoxal in the absence and presence of FMN and pyridoxal 5′-phosphate are listed in Table III. Although our amino acid analyses were not precise enough to determine an exact number of modified residues out of the 40 arginyls present in native monomer, data from these analyses agree with those from incorporation of [^{14}C]phenylglyoxal. Among ~6 modifiable arginyl residues, only ~1 is protected by pyridoxal 5′-phosphate and appears to be essential for enzymatic activity. These data also clearly confirm the stoichiometry of two phenylglyoxal molecules per arginine. Another informative observation is that the number of arginyl residues modified at a given time for samples incubated in the absence and presence of coenzyme FMN is very similar, even though rates of inactivation are quite different in the two cases. It seems likely that FMN binding to apoenzyme causes enhancement of reactivity of the same essential arginyl residue toward reagent.

Discussion

The data presented in this paper support the conclusion that the inactivation of pyridoxamine-5′-phosphate oxidase by arginine reagents is due to modification of an essential arginyl residue located at the substrate/product binding site. The oxidase is inactivated by two arginine reagents, phenylglyoxal and 2,3-butanedione, and an arginine–lysine reagent, 2,4-pentanedione. With these reagents, complete inactivation of the enzyme can be achieved, and the inactivation reaction follows pseudo-first-order kinetics and is first order with respect to reagent, indicating that the inactivation proceeds in an all-or-none fashion.

Phenylglyoxal, shown to be highly selective for the the modification of arginyl residues in proteins (Takahashi, 1968), rapidly inactivates the oxidase in a highly selective manner. Amino acid analyses of the phenylglyoxal-modified enzyme reveal that only arginyl residues are lost; lysyl and histidyl residues remain constant throughout the course of inactivation. Sulfhydryl modification could conceivably cause inactivation by formation of thiohemiacetals (Schubert, 1935; Takahashi, 1968); however, it has been shown that inactivation of the oxidase by modification of a cysteinyl residue is prevented by FMN (Tsuge & McCormick, 1980). The fact that FMN not only does not afford protection but even enhances the rate of inactivation rules out modification of cysteinyl residues as the cause of inactivation by phenylglyoxal. Moreover, the extent of inactivation brought about by this reagent is directly related to the number of arginyl residues modified (cf. Figure 5). 2,3-Butanedione is even more selective for the modification of arginyl residues in proteins. The modification reaction is enhanced by borate buffer, which probably stabilizes the cis-amino–carbinol adduct formed between guanidino group and butanedione, and the modification is reversible by removal of excess butanedione and borate (Riordan, 1973; Borders & Riordan, 1975). With the oxidase, inactivation by butanedione is augmented by borate and fully reversible on removal of borate, as is consistent with the mechanism for arginine modification proposed by Riordan (1973). 2,4-Pentanedione reacts with both arginyl and lysyl residues in proteins, but modified lysyl residues are easily regenerated by treatment of modified protein with hydroxylamine (Gilbert & O'Leary, 1975). Pentanedione inactivates the oxidase. Treatment of inactivated enzyme with hydroxylamine does not restore the enzymatic activity, indicating that the inactivation is due to modification of arginyl and not lysyl residues. The rate at which pentanedione reacts with arginyl residue(s) in the oxidase is about 30 times faster than the corresponding rate with free arginine under similar conditions (Gilbert & O'Leary, 1975). Together, the results strongly suggest that the modification of an essential arginyl residue is fully responsible for the inactivation of the oxidase. One could presume that the conformational change, aggregation, or dissociation to subunits induced by modification of arginyl residues might cause inactivation of the oxidase, but the kinetic data coupled to the fact that modified apoenzyme can bind FMN as well as native enzyme in 1:1 stoichiometry eliminates such possiblities. The importance of anionic phosphate groups for binding of both coenzyme and substrates suggest that cationic arginyl residues could be suitable candidates for such associations in the oxidase. Although FMN does not provide protection against inactivation of the oxidase and inactivated enzyme still binds coenzyme, substrate-competitive inhibitors and product effectively protect the holoenzyme. The extent of protection by the substrate-like inhibitors and product is linearly dependent on their binding constant, and complete protection can be effected at saturation level. These results rule out a nonspecific cause for the protection, such as reaction of inhibitors with phenylglyoxal, and are consistent with the fact that the inhibitors bind well to holoenzyme but not to apoenzyme (Merrill et al., 1980). The protection experiments with competitive inhibitors clearly demonstrate, therefore, that inactivation of the oxidase is due to modification of an essential arginyl residue involved in substrate/product binding. The possibility that a conformational change induced by binding of inhibitor causes the essential arginyl residue to become inaccessible is unlikely, because the inhibitors completely protect the holoenzyme and enzyme inactivated in the absence of inhibitors is not able to bind pyridoxal 5′-phosphate.

In addition to the chemical determinations (both with [^{14}C]phenylglyoxal and by amino acid analyses), kinetic analysis, determined to be first order with respect to reagent, also supports the contention for modification of one arginyl residue at the active site. Although there is a certain limitation in applying kinetic analysis to determine reaction order

(Hayman & Coleman, 1978), the method has been successfully employed previously by several workers (Levy et al., 1963; Marcus et al., 1976; Vensel & Kantrowitz, 1980).

Binding of coenzyme notably enhances the rate of inactivation by both phenylglyoxal and pentanedione. A similar phenomenon with modification of isocitrate dehydrogenase by butanedione was reported by Ehrlich & Coleman (1977). This implies that conversion of apo- to holoenzyme, with exposure of an essential arginyl residue, allows binding of substrate. It is known that binding of coenzyme to apoenzyme generates a substrate/product binding site sufficient to accommodate bulky artificial substrates with K_m values similar to that of natural substrates (Kazarinoff & McCormick, 1975; DePecol & McCormick, 1980).

In a number of enzymes, arginyl residues have been shown to be essential [Vensel & Kantrowitz, (1980) and references cited therein]. In all these cases, the essential arginine has been postulated to interact with the phosphate moiety of the substrate and coenzyme. This has been confirmed by X-ray crystallographic studies of staphylococcal nuclease (Arnone et al., 1971), lactate dehydrogenase (Adams et al., 1973), and horse liver alcohol dehydrogenase (Eklund et al., 1974). It is also known that the structure of the guanidino group is remarkably well suited to interact with a phosphate group (Cotton et al., 1973). Therefore, it seems most likely that in pyridoxamine-5′-phosphate oxidase an arginyl residue at the substrate binding site directly interacts with the phosphate moiety of the substrate. Finally it is worth considering the treatment of oxidase with such excess reagent, e.g., phenylglyoxal, as effects complete inactivation causes modification of only a few of the total of 40 arginyl residues present. Since there are no disulfide bridges, only ionic and hydrophobic interactions can account for the association between the two polypeptide chains which constitute the catalytically active monomer (Tsuge & McCormick, 1980). It is possible that some of the arginyl residues are involved in ionic association at this level. In this connection, it has been shown that the activity of the oxidase is quite sensitive to salts and chaotropic solutes that alter (loosen) the protein–protein interactions (Horiike et al., 1979a).

Acknowledgments

We thank Dr. D. Bowers-Komro and M. Wilhelmi for their assistance in purification of the enzyme and S. Howard for the amino acid analyses.

References

Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossman, M. G., Smiley, I. E., Allison, W. S., Everse, J., Kaplan, N. O., & Taylor, S. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1968–1972.

Arnone, A., Bier, C. J., Cotton, F. A., Day, V. W., Hazen, E. E., Jr., Richardson, D. C., Richardson, J., & Yonath, A. (1971) *J. Biol. Chem. 246*, 2302–2316.

Borders, C., & Riordan, J. F. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 647.

Cotton, F. A., Hazen, E. E., Day, V. W., Larsen, S., Norman, J. G., Wong, S. T. K. & Johnson, K. H. (1973) *J. Am. Chem. Soc. 95*, 2367–2369.

Daemen, F. J. M., & Riordan, J. F. (1974) *Biochemistry 13*, 2865–2871.

DePecol, M. E., & McCormick, D. B. (1980) *Anal. Biochem. 101*, 435–441.

Ehrlich, R. S., & Colman, R. F., (1977) *Biochemistry 16*, 3378–3383.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., & Brändén, C.-I. (1974) *FEBS Lett. 44*, 200–204.

Foster, M., & Harrison, J. H. (1974) *Biochem. Biophys. Res. Commun. 58*, 263–267.

Gilbert, H. F., III, & O'Leary, M. H. (1975) *Biochemistry 14*, 5194–5198.

Gornall, A. G., Bardawill, C. H., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.

Hayman, S., & Colman, R. F. (1978), *Biochemistry 17*, 4161–4168.

Heyl, D., Luz, E., Harris, S. A., & Folkers, K. (1951) *J. Am. Chem. Soc. 73*, 3430–3439.

Horiike, K., & McCormick, D. B. (1980) *J. Theor. Biol. 84*, 691–708.

Horiike, K., Merrill, A. H., Jr., & McCormick, D. B. (1979a) *Arch. Biochem. Biophys. 195*, 325–335.

Horiike, K., Tsuge, H., & McCormick, D. B. (1979b) *J. Biol. Chem. 254*, 6638–6643.

Kazarinoff, M. N., & McCormick, D. B. (1973) *Biochem. Biophys. Res. Commun. 52*, 440–446.

Kazarinoff, M. N., & McCormick, D. B. (1974) *Biochim. Biophys. Acta 359*, 282–287.

Kazarinoff, M. N., & McCormick, D. B. (1975) *J. Biol. Chem. 250*, 3436–3442.

Korytnyk, R. G., & Ikawa, M. (1970) *Methods Enzymol. 18A*, 524–566.

Korytnyk, W., Lachmann, B., & Angelino, N. (1972) *Biochemistry 11*, 722–728.

Lange, L. G., III, Riordan, J. R., & Vallee, B. L. (1974) *Biochemistry 13*, 4361–4370.

Levy, H. M., Leber, P. D., & Ryan, E. M. (1963) *J. Biol. Chem. 238*, 3654–3659.

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

Marcus, F., Schuster, S. M., & Lardy, H. A. (1976) *J. Biol. Chem. 251*, 1775–1780.

McCormick, D. B. (1977) *Photochem. Photobiol. 26*, 169–182.

McCormick, D. B., Kazarinoff, M. N., & Tsuge, H. (1976) in *Flavins and Flavorproteins* (Singer, T. P., Ed.) pp 708–719, Elsevier, Amsterdam.

McCormick, D. B., & Merrill, A. H., Jr. (1980) in *Vitamin B_6 Metabolism and Role in Growth* (Tyfiates, G. P., Ed.) pp 1–26, Food & Nutrition Press, Westport, CT.

Merrill, A. H., Horiike, K., & McCormick, D. B. (1978) *Biochem. Biophys. Res. Commun. 83*, 984–990.

Merrill, A. H., Kasai, S., Matsui, K., Tsuge, H., & McCormick, D. B. (1979a) *Biochemistry 18*, 3635–3641.

Merrill, A. H., Kazarinoff, M. N., Tsuge, H., Horiike, K., & McCormick, D. B. (1979b) *Methods Enzymol. 62*, 563–574.

Merrill, A. H., Jr., Korytnyk, W., Horiike, K., & McCormick, D. B. (1980) *Biochim. Biophys. Acta 626*, 57–63.

Moffatt, J. G., & Khorana, H. G. (1958) *J. Am. Chem. Soc. 80*, 3756–3761.

Riley, H. A., & Gray, A. R. (1943) *Org. Synth. 2*, 509–511.

Riordan, J. F. (1973) *Biochemistry 12*, 3915–3923.

Riordan, J. F., McElvany, K. D., & Borders, C. L., Jr. (1977) *Science (Washington, D.C.) 195*, 884–885.

Rohrbach, M. D., & Bodley, J. W. (1977) *Biochemistry 16*, 1360–1363.

Schubert, M. P. (1935) *J. Biol. Chem. 111*, 671–678.

Snell, E. E., & Haskell, B. M. (1971) *Comp. Biochem. 21*, 47–71.

Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171–6179.
Tsibris, J. C. M., McCormick, D. B., & Wright, L. D. (1966) *J. Biol. Chem. 241*, 1138–1143.
Tsuge, H., & McCormick, D. B. (1980) in *Flavins and Flavoproteins* (Yamano, T., & Yagi, K., Ed.) pp 517–527, Japan Scientific Societies Press, Tokyo.
Vensel, L. A., & Kantrowitz, E. R. (1980) *J. Biol. Chem. 255*, 7306–7310.
Wada, H., & Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089–2095.
Yang, I., Khomutov, R. M., & Metzler, D. E. (1974) *Biochemistry 13*, 3877–3884.

Influence of Ether Linkage on the Lamellar to Hexagonal Phase Transition of Ethanolamine Phospholipids†

J. M. Boggs,* D. Stamp, D. W. Hughes, and C. M. Deber

ABSTRACT: Phosphatidylethanolamine isolated from several sources has been reported to go into the hexagonal phase below physiological temperature, suggesting that a transition to a nonlamellar phase may play a role in membrane function. In the present study, we determined the temperature of the lamellar to hexagonal (L → H) phase transition of bovine white matter ethanolamine phospholipid to be 18 °C. Comparison of this temperature and the hydrocarbon chain composition of bovine white matter ethanolamine phospholipid (77% 1-alk-1-enyl-2-acyl- and 7% 1-alkyl-2-acylglycerophosphoethanolamine) with values reported for phosphatidylethanolamine from other tissues suggested that the alk-1-enyl and alkyl chains on the 1 position of glycerophosphoethanolamine destabilize the lamellar phase. Since this effect could be due either to the double bond between the first and second carbons of the alk-1-enyl chain and/or to the ether linkage, we examined these possibilities using synthetic saturated alkyl and acyl analogues of phosphatidylethanolamine. The temperature of the L → H phase transition was found to increase in the order dialkyl < 1-alkyl-2-acyl ≪ diacyl, while the temperature of the gel to liquid-crystalline (G → L) phase transition increased in the reverse order. Although the ether linkage results in a significant lowering of the temperature of the L → H phase transition, it has little effect on the temperature, enthalpy, and entropy of the G → L phase transition and on the amplitude of motion of fatty acid spin-labels in the gel or liquid-crystalline phases. This indicates that the ether linkage causes only a small increase in the strength of intermolecular hydrogen-bonding interactions between the lipid head groups. This small increase is sufficient to promote the transition to the hexagonal phase, however, which suggests that these interactions for phosphatidylethanolamine have a greater effect on the stability of the hexagonal phase than on the stability of the lamellar gel phase. The presence in myelin of a lipid with a structural modification which destabilizes the lamellar phase suggests that the hexagonal phase may play a role in myelin structure and/or function.

The myelin membrane contains a high content of plasmalogen; 30% of the total phospholipid, mostly the ethanolamine phospholipid fraction (EP)[1] is in this form. The ethanolamine fraction has been found to be 77% 1-alkyl-1-enyl-2-acylglycerophosphoethanolamine (GPE), 7% 1-alkyl-2-acyl-GPE, and 16% diacylphosphatidylethanolamine (PE) (O'Brien & Sampson, 1965; Sun & Horrocks, 1970; Frosolono & Marsh, 1973). A major portion of the 1-alk-1-enyl-2-acyl-GPE of myelin is unusual in that 18:1 is the principal hydrocarbon chain in both the 1 and 2 positions; this species has been reported to be specific for nervous tissue (Sun & Horrocks, 1970; Hack & Helmy, 1977). A high content of plasmalogen lipids is also found in erythrocytes (35% of EP) (Ways & Hanahan, 1964), heart mitochondria (40–60% of EP) (Fleischer et al., 1967; Getz et al., 1968), mouse brain mitochondria (36% of EP) (Sun & Horrocks, 1970), sheep kidney and adrenal cortex (40–60% of EP) Getz et al., 1968), and spermatozoa (64% of total phospholipid and 89% of EP) (Evans et al., 1980).

Plasmalogens have been reported to be more resistant to hydrolysis by phospholipase A_2 than diacyl lipids (Paltauf et al., 1971), but they have other unique properties as well. The ether linkage in analogues of phosphatidylcholine (PC) has been reported to have no effect on lipid fluidity (Schwartz & Paltauf, 1977). However, the phase transition temperature is 2–5 °C higher for the dialkyl analogues of PE, PC (Vaughan & Keough, 1974), and phosphatidic acid (PA) (Blume & Eibl, 1979; Harlos et al., 1979) than for the diacyl analogues while the 1-alkyl-2-acyl analogue of PC has been reported to have a lower phase transition temperature than that of the diester form (Lee & Fitzgerald, 1980).

It has been shown that PE isolated from a number of biological membranes can undergo a lamellar to hexagonal phase transition at temperatures below the physiological temperature

† From The Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8. *Received January 2, 1981.* This investigation was supported by the Multiple Sclerosis Society and the Medical Research Council of Canada. J.M.B. is the recipient of a Career Development Award from the Multiple Sclerosis Society. D.W.H. is a recipient of a postdoctoral fellowship from the Multiple Sclerosis Society.

[1] Abbreviations used: EP, ethanolamine phospholipid fraction; PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; GPE, 1,2-dialkyl or 1-alkyl-2-acyl derivative of glycerophosphoethanolamine; PE(PC), phosphatidylethanolamine prepared from egg phosphatidylcholine; DMPE, 1,2-dimyristoylphosphatidylethanolamine; DPPE, 1,2-dipalmitoylphosphatidylethanolamine; DSPE, 1,2-distearoylphosphatidylethanolamine; DHPE, 1,2-dihexadecylglycerophosphoethanolamine; HPPE, 1-hexadecyl-2-palmitoylglycerophosphoethanolamine; DOPE, 1,2-dioleoylphosphatidylethanolamine; DEPE, 1,2-dielaidoylphosphatidylethanolamine; PG, phosphatidylglycerol; DTPG, 1,2-ditetradecylphosphatidylglycerol; DMPG, 1,2-dimyristoylphosphatidylglycerol; DMPC, 1,2-dimyristoylphosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; ESR, electron spin resonance; NMR, nuclear magnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

0006-2960/81/0420-5728$01.25/0