Liu, H. W., Auchus, R., & Walsh, C. (1984) *J. Am. Chem. Soc. 106*, 5335.
Lussen, K., Naumann, K., & Schroder, R. (1979) *Z. Pflanzenphysiol. 92*, 285.
Martinez-Carrion, M., Tiemeier, D., & Peterson, D. (1970) *Biochemistry 9*, 2574.
Metzler, C. M., Cahill, A., & Metzler, D. E. (1980) *J. Am. Chem. Soc. 102*, 6075.
Morrison, J. F. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 102.
Morrison, J. F., & Stone, S. R. (1985) *Comments Mol. Cell. Biophys. 2*, 347.
Morrison, J. F., & Walsh, C. T. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol.* (in press).
O'Leary, M. H. (1971) *Biochim. Biophys. Acta, 242*, 484.
Peiser, G. D., Wang, T. T., Hoffman, N. E., Yang, S. F., Liu, H. W., & Walsh, C. T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3059.
Pirrung, M. C. (1983) *J. Am. Chem. Soc. 105*, 7207.
Roise, D., Soda, K., Yagi, T., & Walsh, C. (1984) *Biochemistry 23*, 5195.
Shaltiel, S., & Cortijo, M. (1970) *Biochem. Biophys. Res. Commun. 41*, 594.
Shapiro, R., & Riordan, J. F. (1984) *Biochemistry 23*, 5234.
Shioiri, T., Ninomiya, K., & Yamada, S. I. (1972) *J. Am. Chem. Soc. 94*, 6203.
Singh, R. K., & Danishefsky, S. (1975) *J. Org. Chem. 40*, 2969.
Walsh, C. (1979) in *Enzymatic Reaction Mechanisms*, pp 828–833, Freeman, San Francisco.
Walsh, C., Pascal, R. A., Jr., Johnston, M., Raines, R., Dikshit, D., Krantz, A., & Honma, M. (1981) *Biochemistry 20*, 7509.
Wertz, D. A., & Allinger, N. C. (1974) *Tetrahedron 30*, 1579.
Wiberg, K. B., & Ellison, G. B. (1974) *Tetrahedron 30*, 1573.
Wiesendanger, R., Martinoni, B., Boller, T., & Arigoni, D. (1986a) *J. Chem. Soc. Commun.*, 238.
Wiesendanger, R., Martinoni, B., & Arigoni, D. (1986b) *Experientia 42*, 207.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324.
Yang, S. F., & Adams, D. O. (1980) in *The Biochemistry of Plants* (Stumpf, P. K., Ed.) Vol. 4, p 163, Academic, New York.
Yang, S. F., & Hoffman, N. E. (1984) *Annu. Rev. Plant Physiol. 35*, 155.

Phosphonate Analogues of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate as Substrates or Inhibitors of Procaryotic and Eucaryotic Enzymes Degrading Dinucleoside Tetraphosphates†

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ABSTRACT: The substrate specificity of procaryotic and eucaryotic AppppA-degrading enzymes was investigated with phosphonate analogues of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA). App(CH_2)ppA (I), App(CHBr)ppA (II), and Appp(CH_2)pA (III), but not Ap(CH_2)pp(CH_2)pA (IV), are substrates for lupin AppppA hydrolase (EC 3.6.1.17) and phosphodiesterase I (EC 3.1.4.1). None of the four analogues is hydrolyzed by bacterial AppppA hydrolase (EC 3.6.1.41), and only analogue III is degraded by yeast AppppA phosphorylase (EC 2.7.7.53). The analogues are competitive inhibitors of all four enzymes. The affinity of analogue IV is 3–40-fold lower than that of analogues I–III for all four enzymes. Introduction of one methylene (as in I and III) [or bromomethylene (as in II)] group into AppppA results in a 3–15-fold increase of its affinity for lupin and *Escherichia coli* AppppA hydrolases. The same modifications only negligibly (10–30%) affect its affinity for yeast AppppA phosphorylase and decrease its affinity for lupin phosphodiesterase I about 2.5-fold. The data provide further evidence for the heterogeneity among catalytic sites of all four AppppA-degrading enzymes.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA), discovered as a product of the back-reaction of aminoacyl-tRNA synthetase (Zamecnik et al., 1968), has subsequently turned out to be a ubiquitous dinucleotide present in all cell types investigated (Zamecnik, 1983; Garrison & Barnes, 1984). The dinucleotide is synthesized in vitro by some aminoacyl-tRNA synthetases from several organisms (Zamecnik et al., 1968; Plateau et al., 1981; Goerlich et al., 1982; Jakubowski, 1983). Presumably the same enzymes are involved in AppppA synthesis in vivo, although this remains to be confirmed. A variety of factors affect the cellular level of AppppA, and on the basis of this, two hypotheses conce ning the function of AppppA in cells have been advanced. One of them proposes that AppppA is a positive pleiotropic activator that modulates DNA

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replication (Rapaport & Zamecnik, 1976), and according to the other, AppppA is a signal nucleotide produced by cells during oxidative stress (Bochner et al., 1984; Baker & Jacobson, 1986). Although the two hypotheses may not be exclusive, recent results obtained with *Physarum polycephalum* are consistent with AppppA being a signal nucleotide of an oxidative stress and do not support the hypothesis that AppppA is a positive pleiotropic activator that modulates DNA replication (Garrison et al., 1986).

The cellular level of AppppA is apparently determined by synthetic activities of aminoacyl-tRNA synthetases and degradative activities of various AppppA hydrolases (Mechulam et al., 1985). Although synthesis of AppppA and related compounds by aminoacyl-tRNA synthetase from the various procaryotic and eucaryotic organisms is rather uniform, each class of organisms uses a unique mechanism of degradation of the dinucleoside oligophosphates. Asymmetrical dinucleoside tetraphosphatase or AppppA hydrolase (EC 3.6.1.17) hydrolyzing AppppA to ATP and AMP has been isolated from higher eucaryotic tissues, animals (Labaton et al., 1975) and plants (Jakubowski & Guranowski, 1983). Symmetrical AppppA hydrolase (EC 3.6.1.41) present in the slime mold *Physarum polycephalum* (Barnes & Culver, 1982) and in bacteria (Guranowski et al., 1983; Plateau et al., 1985) hydrolyzes AppppA to ADP. In yeast, AppppA is cleaved phosphorolytically to ATP and ADP by AppppA α,β-phosphorylase (EC 2.7.7.53) (Guranowski & Blanquet, 1985). Eucaryotic phosphodiesterases (Jakubowski & Guranowski, 1983; Camselle et al., 1984; Luthie & Ogilvie, 1985) and nucleotide pyrophosphatase (EC 3.6.1.9) (Bartkiewicz et al., 1984) hydrolyze AppppA to AMP and ATP. A variety of dinucleoside oligophosphates are degraded by single enzymes in procaryotes and *P. polycephalum*, but in higher eucaryotes the function is divided between two specific enzymes, dinucleoside tetraphosphatase and dinucleoside triphosphatase (Jakubowski & Guranowski, 1983; Sillero et al., 1977). Although various AppppA-degrading enzymes perform the same function, i.e., degradation of AppppA, their active sites seem not to be structurally related.

We have begun using phosphonate analogues of AppppA to study AppppA-degrading enzymes from procaryotes and eucaryotes with two objectives in mind. First, we might be able to learn what structural features of the polyphosphate chain of AppppA are essential for binding and catalysis with various AppppA-degrading enzymes. This may aid us in further mapping of their catalytic sites. Second, nondegradable analogues of AppppA that still retain the ability to bind to an AppppA-degrading enzyme might provide valuable tools in studies of the function and metabolism of AppppA. By specific inhibition of AppppA-degrading enzymes in vivo [by introducing a nonhydrolyzable analogue of AppppA into permeabilized cells or intact cells, e.g., by injection into oocytes (Guedon et al., 1985)], we may be able to manipulate cellular levels of AppppA. As a first step toward these objectives we describe interactions of recently synthesized phosphonate analogues of AppppA (Tarussova et al., 1983, 1985) with several pure AppppA-degrading enzymes of procaryotic and eucaryotic origin. The phosphonate analogues of AppppA are potent inhibitors of microbial AppppA-degrading enzymes.

Materials and Methods

Chemicals. Di[8-^{3}H]adenosine tetraphosphate (555 GBq/mmol) was purchased from Amersham. Unlabeled AppppA, ATP (pppA), ADP (ppA), AMP (pA), adenosine 5′-(β,γ-methylenetriphosphate) [p(CH_2)ppA], adenosine 5′-(α,β-methylenetriphosphate) [pp(CH_2)pA], and adenosine 5′-(α,β-methylenediphosphate) [p(CH_2)pA] were from Sigma.

Preparation of Phosphonate Analogues of AppppA. The phosphonate analogues of AppppA were synthesized and purified on a DEAE-cellulose (Whatman) column as described earlier (Tarussova et al., 1983, 1985). Analogue III was additionally purified by high-performance liquid chromatography (HPLC) with a C_{18} reverse-phase column. The analogues were homogeneous as determined by thin-layer chromatography (TLC) analysis with the two chromatographic systems described below.

Chromatographic Systems. System I was poly(ethyleneimine)–cellulose sheets (from E. Merck) developed first for 20 min in 75% methanol and then for 1 h in 0.85 M LiCl. System II was silica gel sheets (E. Merck) developed for 2 h in dioxane/ammonia/water (6:1:4 v/v). System I was used routinely in all the enzyme assays (see below). System II was particularly useful for analyzing the incubation mixtures in which the phosphonate analogues of AppppA were tested as potential substrates of the AppppA-degrading enzymes.

Enzymes. Asymmetrical AppppA hydrolase and phosphodiesterase I were isolated from yellow lupin (*Lupinus luteus*) seeds as described previously (Jakubowski & Guranowski, 1983). *E. coli* symmetrical AppppA hydrolase and yeast AppppA α,β-phosphorylase were purified according to Guranowski et al. (1983) and Guranowski and Blanquet (1985), respectively. Stock solutions of the enzymes were diluted before use with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/KOH (pH 8.2) containing 10 μM dithiothreitol, 10% glycerol and 2 mg/mL bovine serum albumin.

Enzyme Assays. Incubation mixtures (50 μL) were slightly modified with respect to those used previously (Jakubowski & Guranowski, 1983; Guranowski et al., 1983; Guranowski & Blanquet, 1985) and contained the following: for phosphodiesterase I, 50 mM Hepes/KOH (pH 8.2), 10 μM dithiothreitol, and 50 μM [8-^{3}H]AppppA (about 250 000 cpm) (mixture A); for asymmetrical AppppA hydrolase, mixture A plus 5 mM $MgCl_2$ (mixture B); for symmetrical AppppA hydrolase, mixture A plus 0.1 mM $CoCl_2$ (mixture C); for AppppA α,β-phosphorylase, mixture B plus 5 mM K_2HPO_4 (mixture D). Reactions were initiated by the addition of rate-limiting amounts of the appropriate enzyme. Incubations were carried out at 30 °C. Velocity of the enzymic reactions was estimated from four experimental points of the time course analysis. At time intervals (usually after 3, 6, 9, and 15 min), the 4-μL aliquots were transferred onto poly(ethyleneimine)–cellulose sheets, the appropriate standards (AppppA plus AMP or AppppA plus ADP) were added, and the chromatograms were developed with system I. Spots of AMP (in the case of asymmetrical AppppA hydrolase and phosphodiesterase I) or ADP (in the case of symmetrical AppppA hydrolase and AppppA phosphorylase) were visualized under ultraviolet light and cut out, and their radioactivity was determined by liquid scintillation counting. For inhibition measurements, the phosphonate analogues of AppppA at concentrations from 0.025 to 1.0 mM were included into the assays. The K_i values for the AppppA analogues were estimated from the Dixon plot (Dixon, 1953). The accuracy of the measurements was ±10%.

Assays of the Hydrolysis of Phosphonate Analogues of AppppA. The rates of degradation of the AppppA analogues by some of the enzymes were estimated from densitometric measurements (at 260 nm using a Vitatron TLD-100 densitometer) of products (AMP or ADP) separated from sub-

Table I: R_f Values for Phosphonate Analogues of AppppA and Products of Their Possible Degradations

compound	R_f values	
	system I[d]	system II[b]
AppppA	0.17	0.61
pA	0.39	0.65
ppA	0.24	0.24
pppA	0.07	0.14
App(CH_2)ppA	0.28	0.49
p(CH_2)ppA	0.25	0.07
App(CHBr)ppA	0.17	0.40
p(CHBr)ppA	nd	0.03
Appp(CH_2)pA	0.20	0.41
pp(CH_2)pA	0.18	0.09
Ap(CH_2)pp(CH_2)pA	0.36	0.33
p(CH_2)pA	0.41	0.19

[a] System I: plastic sheets precoated with pooly(ethylenimine)–cellulose (from E. Merck) developed for 20 min in 75% methanol followed by 1 h in 0.85 M LiCl. [b] System II: aluminum sheets precoated with silica gel (from E. Merck) developed for 2 h in dioxane/ammonia/water (6:1:4 v/v).

strates in system II. Incubation mixtures were as described above except 0.5 mM AppppA or its analogue was used instead of 50 μM [^{3}H]AppppA.

Results

Are Phosphate Analogues of AppppA Substrates for AppppA-Degrading Enzymes? The following phosphonate analogues of AppppA were used in these studies: I, with $-CH_2-$ group between P^2 and P^3 [App(CH_2)ppA]; II, with –CHBr– group between P^2 and P^3 [App(CHBr)ppA]; III, with $-CH_2-$ group between P^1 and P^2 [Appp(CH_2)pA]; IV, with two $-CH_2-$ groups, one between P^1 and P^2 and the other between P^3 and P^4 [Ap(CH_2)pp(CH_2)pA]. Three AppppA-degrading enzymes (lupin AppppA hydrolase, phosphodiesterase I, and yeast AppppA phosporylase) of the four studied split the anhydride bond between P^1 and P^2, and one enzyme (*E. coli* AppppA hydrolase) splits the anhydride bond between P^2 and P^3 in unmodified AppppA molecules. Accordingly, the expectation was that analogues I–III (but not analogue IV) may possibly be substrates for the two lupin enzymes and the yeast enzyme. The *E. coli* enzyme was expected to hydrolyze analogues III and IV but not analogues I and II.

Incubation mixtures containing 0.5 mM analogue and appropriate enzyme (at concentration sufficient to completely degrade 0.5 mM AppppA within 1 min) were maintained at 30 °C for up to 16 h. Aliquots of incubation mixtures were analyzed by one-dimensional TLC with system II. The system clearly separates the phosphonate analogues of AppppA from possible products of their breakdown as determined in preliminary experiments (for the R_f values, see Table I). As expected, analogues I–III (but not analogue IV) were hydrolyzed by the two lupin enzymes. AMP and adenosine 5′-(β,γ-methylenetriphosphate) (with analogue I), AMP and adenosine 5′-[β,γ-(bromomethylene)triphosphate] (with analogue II), or AMP and adenosine 5′-(α,β-methylenetriphosphate) with analogue III were the products. With lupin phosphodiesterase the rates of hydrolysis of I–III were comparable with the rate of hydrolysis of AppppA. With lupin AppppA hydrolase the rates of hydrolysis of I–III were 7–50-fold slower than the rate of hydrolysis of AppppA (Table II). This result is not unexpected when one considers much wider substrate specificity of the lupin phosphodiesterase as compared to rather strict substrate specificity of lupin AppppA hydrolase (Jakubowski & Guranowski, 1983). Also as expected, analogue III was degraded by yeast AppppA phosphorylase, albeit at only 1% of the rate observed with AppppA (Table II).

Table II: Kinetic Indices for AppppA and Phosphonate Analogues with Procaryotic and Eucaryotic AppppA-Degrading Enzymes

dinucleotide	V_{rel}	K_m or K_i (μM)	relative specificity[a]
E. coli AppppA Hydrolase			
AppppA	1.0	25	1
App(CH_2)ppA	0.00	3.3	
App(CHBr)ppA	0.00	1.7	
Appp(CH_2)pA	0.00	2.8	
Ap(CH_2)pp(CH_2)pA	0.00	8.1	
Lupin AppppA Hydrolase			
AppppA	1.0	1.0	1
App(CH_2)ppA	0.06	0.25	0.24
App(CHBr)ppA	0.16	0.32	0.50
Appp(CH_2)pA	0.02	0.65	0.03
Ap(CH_2)pp(CH_2)pA	0.00	10.9	
Lupin Phosphodiesterase I			
AppppA	1.0	2.0	1
App(CH_2)ppA	1.3	4.6	0.57
App(CHBr)ppA	1.65	5.0	0.66
Appp(CH_2)pA	1.0	3.7	0.54
Ap(CH_2)pp(CH_2)pA	0.00	66	
Yeast AppppA Phosphorylase			
AppppA	1.0	60	1
App(CH_2)ppA	0.00	44	
App(CHBr)ppA	0.00	69	
Appp(CH_2)pA	0.01	24	0.025
Ap(CH_2)pp(CH_2)pA	0.00	196	

[a] Relative specificity, defined as relative value of k_{cat}/K_m, has been calculated according to Fersht (1974). It has been assumed that $K_i = K_m$.

Contrary to predictions, the yeast AppppA phosphorylase did not degrade analogues I and II, and the *E. coli* AppppA hydrolase did not degrade analogues III and IV, even after 16-h incubation. Under the conditions used, degradation at rates as slow as 0.01% of that observed with AppppA would be detectable. In agreement with predictions, analogues I and II were not degraded by the *E. coli* enzyme, and analogue IV was not degraded by the yeast and lupin enzymes. As shown below, all analogues do interact with each one of the enzymes, with affinities either decreased (up to 30-fold), increased (up to 15-fold), or not changed significantly.

K_i Values for Phosphonate Analogues of AppppA. All four analogues of AppppA are competitive inhibitors of the two microbial AppppA-degrading enzymes. Analogue IV is an inhibitor of the two lupin AppppA-degrading enzymes, and as shown above, analogues I–III are substrates. However, the affinity of analogues I–III for the lupin enzymes can also be determined from the apparent inhibition of [^{3}H]AppppA hydrolysis by the analogues I–III. The K_i values, calculated from the Dixon plots, are presented in Table II. Also given in Table II are published K_m values for AppppA (Jakubowski & Guranowski, 1983; Guranowski et al., 1983; Guranowski & Blanquet, 1985). Of the three phosphonate analogues of AppppA, analogue IV is the weakest inhibitor with all four enzymes studied. The *E. coli* AppppA hydrolase is the most susceptible to inhibition exerted by the phosphonate analogues of AppppA. The K_i values for all four analogues were 3–10-fold lower than the K_m value (25 μM) for AppppA (Guranowski et al., 1983). Analogues I–III are bound 1.6–4-fold better than AppppA by lupin AppppA hydrolase. Analogue IV is bound much weaker than AppppA by lupin AppppA hydrolase and phosphodiesterase and by yeast AppppA phosphorylase. Analogues I and II bind 2.5-fold less strongly than AppppA to the lupin phosphodiesterase and with affinity

similar to that for the binding of AppppA to the yeast AppppA phosphorylase.

Discussion

Much of our understanding of biochemical pathways come from the studies that utilized specific inhibitors of the particular steps of a pathway. In cases where no genetic methods were available to determine the function of a particular protein, design and application of appropriate inhibitors of that function was the approach of choice. We are attempting to use this approach toward the end of gaining insights into the role(s) of AppppA in cellular physiology both in procaryotes (Guranowski et al., 1983) and in eucaryotes (Jakubowski & Guranowski, 1983; Guranowski & Blanquet, 1985). Along these lines several new phosphonate analogues of AppppA were synthesized (Tarussova et al., 1983, 1985), and as described in this paper, their interactions with several AppppA-degrading enzymes of various origin were studied.

Each of the four AppppA-degrading enzymes interacts with the common substrate AppppA in a distinct way. Each enzyme recognizes different features of the substrate and acts upon either one of the two kinds of the pyrophosphate bonds (P^1–O–P^2 or P^2–O–P^3). In this study we have demonstrated that modification of specific sites in the polyphosphate chain of AppppA may lead to total loss of substrate properties but not of its ability to bind to an enzyme. With lupin AppppA hydrolase and phosphodiesterase I, analogues I–III, but not IV, are substrates. Thus, with the two lupin AppppA–hydrolyzing enzymes, modification of the two possible sites of the reaction in the substrate (as in analogue IV), but not of the adjacent site (as in analogues I and II), prevents the substrate from reacting. However, modification of just one of the two possible sites of reaction (as in analogue III) does not prevent analogue III from the reaction with the two lupin enzymes and also with the yeast enzyme. Bacterial AppppA hydrolase does not use any of the four phosphonate analogues of AppppA as substrates. Yeast AppppA phosphorylase does not use analogues I, II, and IV as substrates. Apparently, with the two microbial enzymes, modification of not only the actual site of reaction (as in analogues I and II for the *E. coli* enzyme or as in analogue IV for the yeast enzyme) but also of adjacent sites (as in analogues III and IV for the *E. coli* enzyme or as in analogues I and II for the yeast enzyme) in the polyphosphate chain of AppppA prevents the substrate from reacting. Lack of degradation of the phosphonate analogues of AppppA suggests that at least one of the adjacent anhydride bond oxygens in addition to the oxygen at the actual site of reaction is essential for catalysis with the *E. coli* and yeast enzymes. This requirement is not shared by the lupin enzymes for which the anhydride bond oxygen adjacent to the site of the reaction in the substrate is not essential.

The phosphonate analogues of AppppA, which are not degraded by the *E. coli* and yeast AppppA-degrading enzymes, all do bind to the two microbial enzymes and are potent inhibitors, in particular of the *E. coli* AppppA hydrolase (with K_i in the micromolar range). Substitution of any of the anhydride bond oxygens by methylene or bromomethylene residues (as in analogues I–IV) in the tetraphosphate chain of AppppA increases its affinity for the *E. coli* enzyme. This may indicate a hydrophobic nature of the AppppA-binding site. Catalysis in this case may possibly be assisted by strain resulting from repulsion of the polyphosphate chain of AppppA from the active site of the *E. coli* enzyme. Releasing the strain by increasing the hydrophobicity of the polyphosphate chain of AppppA (as in analogues III and IV, which are expected to be hydrolyzed, but are not) abolishes catalysis. Alternatively, the phosphonate analogues of AppppA may be transition-state analogues for the *E. coli* enzyme. The reasons for the lack of catalysis in the case of yeast AppppA phosphorylase and analogues I and II seem to be different. Increase in hydrophobicity of the polyphosphate chain of AppppA (by introducing modifications as in analogues I and II) does not have a significant effect on its binding by the yeast AppppA phosphorylase.

In our earlier studies in which we (Jakubowski & Guranowski, 1983; Guranowski et al., 1983; Guranowski & Blanquet, 1985) examined effects of nucleoside substitution or removal and the effects of changing the numbers of phosphate residues in the polyphosphate chain of AppppA on the catalysis, we have shown that each of the four AppppA-degrading enzymes exhibits distinct substrate specificity and, therefore, has a unique active site. In this study we have further probed the nature of the active site of these enzymes by using phosphonate analogues of AppppA and have shown the heterogeneity of the active sites with respect to their interactions with the tetraphosphate part of the substrate molecule.

Finally, the results presented in this paper also identify phosphonate analogues of AppppA as antimetabolites that can inactivate AppppA-degrading enzymes from procaryotic and eucaryotic sources, thereby providing possibly useful tools in studies of the function and metabolism of AppppA. To this end it is interesting to note that all four phosphonate analogues of AppppA are only weak inhibitors ($K_i \sim 1$ mM) of aminoacyl-tRNA synthetases (Biryukov et al., 1987), which indicates that AppppA-degrading enzymes may be specific targets for inhibition in vivo.

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References

Baker, J. C., & Jacobson, M. K. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2350–2352.

Barnes, L. D., & Culver, C. A. (1982) *Biochemistry 21*, 6123–6128.

Bartkiewicz, M., Sierakowska, H., & Shugar, D. (1984) *Eur. J. Biochem. 143*, 419–426.

Biryukov, A. I., Tarussova, N. B., & Khomutov, R. M. (1987) *Bioorg. Khim.* (in press).

Bochner, B. R., Lee, Ph. C., Wilson, S. W., Cutler, C. W., & Ames, B. N. (1984) *Cell (Cambridge, Mass.) 37*, 225–232.

Cameselle, J. C., Costas, M. J., Sillero, M. A. G., & Sillero, A. (1984) *J. Biol. Chem. 259*, 2879–2885.

Dixon, M. (1953) *Biochem. J. 55*, 170–171.

Fersht, A. R. (1974) *Proc. R. Soc. London, B 187*, 397–407.

Garrison, P. N., & Barnes, L. D. (1984) *Biochem. J. 217*, 805–811.

Garrison, P. N., Mathis, S. A., & Barnes, L. D. (1986) *Mol. Cell. Biol. 6*, 1179–1186.

Goerlich, O., Foeckler, R., & Holler, E. (1982) *Eur. J. Biochem. 126*, 135–142.

Guedon, G., Sovia, D., Ebel, J. P., Befort, N., & Remy, P. (1985) *EMBO J. 4*, 3743–3749.

Guranowski, A., & Blanquet, S. (1985) *J. Biol. Chem. 260*, 3542–3547.

Guranowski, A., Jakubowski, H., & Holler, E. (1983) *J. Biol. Chem. 258*, 14784–14789.

Jakubowski, H. (1983) *Acta Biochim. Pol. 30*, 51–69.

Jakubowski, H., & Guranowski, A. (1983) *J. Biol. Chem. 258*, 9982–9989.

Lobaton, C. D., Vallejo, C. G., Sillero, A., & Sillero, M. A.

G. (1975) *Eur. J. Biochem. 50*, 495–501.
Luthje, J., & Ogilvie, A. (1985) *Eur. J. Biochem. 149*, 119–121.
Mechulam, Y., Fromant, M., Mellot, P., Plateau, P., Blanchin-Roland, S., Fayat, G., & Blanquet, S. (1985) *J. Bacteriol. 164*, 63–69.
Plateau, P., Mayaux, J.-F., & Blanquet, S. (1981) *Biochemistry 20*, 4654–4662.
Plateau, P., Fromant, M., Brevet, A., Gesquiere, A., & Blanquet, S. (1985) *Biochemistry 24*, 914–922.
Rapaport, E., & Zamecnik, P. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3984–3988.
Sillero, M. A. G., Villalba, R., Moreno, A., Quintanilla, M., Lobaton, C. D., & Sillero, A. (1977) *Eur. J. Biochem. 76*, 331–337.
Tarussova, N. B., Shumiyanzeva, V. V., Krylov, A. C. Karpeisky, M. Ya., & Khomutov, R. M. (1983) *Bioorg. Khim. 9*, 838–843.
Tarussova, N. B., Zavgorodny, C. G., & Osipova, T. I. (1985) *Bioorg. Khim. 11*, 802–807.
Zamecnik, P. (1983) *Anal. Biochem. 134*, 1–10.
Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., & Randerath, K. (1966) *Biochem. Biophys. Res. Commun. 24*, 91–97.

Solvent and Solvent Proton Dependent Steps in the Galactose Oxidase Reaction†

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ABSTRACT: Solvent and solvent proton dependent steps involved in the mechanism of the enzyme galactose oxidase have been examined. The deuterium kinetic solvent isotope effect (KSIE) on the velocity of the galactose oxidase catalyzed oxidation of methyl β-galactopyranoside by O_2 was measured. Examination of the thermodynamic activation parameters for the reaction indicated that the isotope effect was attributable to a slightly less favorable $\Delta H^{\ddagger}$ value, consistent with a KSIE on proton transfer. A detailed kinetic analysis was performed, examining the effect of D_2O on the rate of reaction over the pH range 4.8–8.0. Both pL–rate profiles exhibited bell-shaped curves. Substitution of D_2O as solvent shifted the pK_{es} values for the enzymic central complex: pK_{es1} from 6.30 to 6.80 and pK_{es2} from 7.16 to 7.35. Analysis of the observed shifts in dissociation constants was performed with regard to potential hydrogenic sites. pK_{es1} can be attributed to a histidine imidazole, while pK_{es2} is tentatively assigned to a Cu^{2+}-bound water molecule. A proton inventory was performed (KSIE = +1.55); the plot of k_{cat} vs. mole fraction D_2O was linear, indicating the existence of a single solvent-derived proton involved in a galactose oxidase rate-determining step (or steps). The pH dependence of CN^- inhibition was also examined. The K_i–pH profile indicated that a group ionization, with pK_a = 7.17, modulated CN^- inhibition; K_i was at a minimum when this group was in the protonated state. The inhibition profile followed the alkaline limit of the pH–rate profile for the enzymic reaction, suggesting that the group displaced by CN^- was also deprotonating above pH 7. Consistent with this suggestion was the D_2O-dependent shift in pK_a (+0.17) of the group modulating CN^- inhibition which was similar to the shift observed in pK_{es2}. Nuclear and electron magnetic resonance studies have shown previously that CN^- coordinates equatorially to the enzymic Cu(II), apparently displacing a water molecule [Marwedel, B. J., Kosman, D.J., Bereman, R. D., & Kurland, R. J. (1981) *J. Am. Chem. Soc. 103*, 2842–2847]. The data indicate that this Cu(II)-bound H_2O is required in the protonated aquo state for catalysis and is responsible for the KSIE observed in the pL–rate profile. A mechanism that couples electron transfer to O_2 with the proton transfer step(s) probed by these experiments is discussed.

Galactose oxidase (EC 1.1.3.9) is a copper-containing enzyme that catalyzes the conversion of a primary alcohol and molecular oxygen to an aldehyde and hydrogen peroxide (Kosman et al., 1974; Ettinger & Kosman, 1981). GO[1] is the only known mononuclear Cu(II) protein that catalyzes a two-electron redox reaction involving molecular oxygen without the involvement of another metal ion or organic cofactor. Because of this unique characteristic and because it is the only known type 2 (nonblue) Cu(II) protein possessing a single such prosthetic group, GO has been of considerable interest both spectrally and mechanistically. These features make GO an experimentally attractive choice for the study of the structure and function of Cu(II)-active sites involved in substrate-level oxidation.

One of the least well-characterized aspects of metal-dependent oxidation is the coupling of the requisite H^+- and electron-transfer steps. GO exhibits a bell-shaped pH dependence for V_{max}. The ascending limb of the pH–rate profile has been assigned to a histidine imidazole (pK_a = 6.3) which may provide base catalysis of abstraction of a substrate carbinol proton (Kwiatkowski et al., 1977). The origin of the descending, alkaline limb has not been elucidated. However, a variety of kinetic and spectral observations indicate that this group ionization could be associated with an equatorially coordinated water molecule. Specifically, ^{17}O ESR measurements show H_2O to coordinate equatorially to the Cu(II) (Melnyk, 1979). This H_2O is displaced by CN^-, known also

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[1] Abbreviations: GO, galactose oxidase; ESR, electron spin resonance; DMF, dimethylformamide.

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