High energy charge requires low activity thus the inhibition by PEP and ATP. Indeed, ATP is a common inhibitor of PFKs from other organisms as well (Uyeda, 1979).

It is not clear from the structure of the active site how ATP is acting. ATP interacts with Arg72, which we have shown is an important mediator of cooperative signals between subunits (Berger & Evans, 1990). In the T state of the *B. stearothermophilus* enzyme, this residue forms a stabilizing salt bridge with Glu241 from the adjacent subunit. It is not clear how ATP could either stabilize this interaction or bind without interfering with it. Model building of the R-state configuration of ATP in the T-state structure reveals no obvious interactions that might account for the ability of ATP to stabilize the T state. It is formally possible that ATP may be acting through a site distinct from the active site. Perhaps a structural determination in the presence of only ATP might be helpful in explaining this.

Registry No. Fru6P, 643-13-0; PEP, 138-08-9; 5′-ADP, 58-64-0; 5′-ATP, 56-65-5; PFK, 9001-80-3.

References

Banas, T., Gontero, B., Drews, V. L., Johnson, S. L., Marcus, F., & Kemp, R. G. (1988) *Biochim. Biophys. Acta 957*, 178–184.

Berger, S. A., & Evans, P. R. (1990) *Nature 343*, 575–576.

Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31*, 13–35.

Hellinga, H. W., & Evans, P. R. (1987) *Nature 327*, 437–439.

Kotlarz, D., & Buc, H. (1977) *Biochim. Biophys. Acta 484*, 35–48.

Kotlarz, D., & Buc, H. (1982) *Methods Enzymol. 90*, 60–70.

Kundrot, C. E., & Evans, P. R. (1991) *Biochemistry 30*, 1478–1484.

Lau, F. K.-T. (1989) Ph.D. Thesis, University of London.

Lau, F. K.-T., & Fersht, A. R. (1987) *Nature 326*, 811–812.

Lau, F. K.-T., Fersht, A. R., Hellinga, H. W., & Evans, P. R. (1987) *Biochemistry 26*, 4143–4148.

Le-Bras, G., & Garel, J.-R. (1985) *J. Biol. Chem. 260*, 13450–13453.

Le-Bras, G., Teschner, W., Deville-Bonne, D., Garel, J. R. (1989) *Biochemistry 28*, 6836–6841.

Press, W. H. (1988) *Numerical Recipes in C: The Art of Scientific Computing*, Cambridge University Press, Cambridge.

Rypniewski, W. R., & Evans, P. R. (1989) *J. Mol. Biol. 207*, 805–821.

Schirmer, T., & Evans, P. R. (1990) *Nature 343*, 140–145.

Schmid, F. X. (1989) in *Protein Structure, A Practical Approach* (Creighton, T. E., Ed.) pp 251–285, IRL Press, Oxford.

Teschner, W., & Garel, J.-R. (1989) *Biochemistry 28*, 1912–1916.

Uyeda, K. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 194–243.

Valdez, B. C., French, B. A., Younathan, E. S., & Chang, S. H. (1989) *J. Biol. Chem. 264*, 131–135.

Rational Design of Quinazoline-Based Irreversible Inhibitors of Human Erythrocyte Purine Nucleoside Phosphorylase†

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ABSTRACT: Described herein is the rational design of irreversible inhibitors of human erythrocyte purine nucleoside phosphorylase (PNPase). Inhibitor design started with the observation that the amino group of 8-aminoquinazolin-4(3*H*)-one interacts with enzyme-bound phosphate. This observation correctly predicted that the 5,8-dione (quinone) and 5,8-dihydroxy (hydroquinone) derivatives of quinazolin-4(3*H*)-ones would enter the active site. The amine–phosphate interaction also served to confirm that a quinazolin-4(3*H*)-one binds in the PNPase active sites like a purine substrate. From models of the PNPase active site it was possible to design quinazoline-based quinones that undergo a reductive-addition reaction with an active-site glutamate residue. The best inhibitor studied, 2-(chloromethyl)quinazoline-4,5,8(3*H*)-trione, rapidly inactivates PNPase by a first-order process with an inhibitor to enzyme stoichiometry of 150. The active-site hydroquinone adduct of this inhibitor eliminates a leaving group to afford a quinone methide species positioned to alkylate another active-site glutamate residue. Thus, this inhibitor is designed to cross-link the PNPase active site by reductive addition followed by the generation of an alkylating quinone methide species.

Purine nucleoside phosphorylase (PNPase) catalyzes the reversible phosphorylase of inosine and guanosine to the respective bases and ribose 1-phosphate. The degradative nature of this enzyme initially suggested that PNPase inhibitors would have only limited chemotherapeutic value (Parks & Agarwal, 1972). In the past ten years, however, it became apparent that PNPase inhibitors could be used in the prevention of foreign tissue rejection, in the treatment of gout and malaria, and for the potentiation of antineoplastic nucleosides (Parks et al., 1981; Kazmers et al., 1981; Daddona et al., 1986; Shewach et al., 1986). Thus, there has been a great deal of effort devoted to the design of PNPase inhibitors, most of which are purine, purine nucleoside, or purine nucleotide analogues

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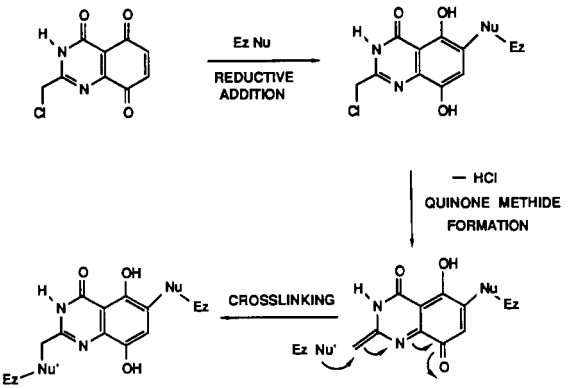
Scheme I



(Jordan & Wu, 1978; Parks et al., 1981; Stoeckler et al., 1982; Salamone & Jordan, 1982; Daddona et al., 1986; Sircar et al., 1986; Nakamura et al., 1986; Stoeckler et al., 1986a,b; Stein et al., 1987; Krenitsky et al., 1990). Described herein is the rational design of a new class of PNPase inhibitors based on the quinazoline ring system. Our best inhibitor, **1** in Scheme I, is capable of rapid irreversible enzyme inhibition at low inhibitor/enzyme stoichiometry. The successful design of this inhibitor shows that quinazolines can be used as starting points for PNPase inhibitor design.

The rational design of a PNPase irreversible inhibitor started with the observation that 8-aminoquinazolin-4(3*H*)-one enters the enzyme active site such that the 8-amino group interacts with enzyme-bound phosphate (Dempcy & Skibo, 1991). This observation correctly predicted that 5,8-dione (quinone) and 5,8-dihydroxy (hydroquinone) derivatives of the quinazoline ring would likewise enter the enzyme active site. The strategy was to utilize an active-site nucleophilic residue to convert quinone **1** to the hydroquinone derivative **2** by reductive addition (Finley, 1974). The resulting hydroquinone would then afford an alkylating quinone methide species by elimination of HCl (Lemus & Skibo, 1988; Lemus et al., 1989) and then trap a second nucleophilic residue (Scheme I). Docking the quinazoline system **1** into the active site of the three-dimensional PNPase structure (Ealick et al., 1990) suggested that the first residue is glutamate 259 and the second residue is glutamate 201. Our studies show that the reductive addition process (**1** → **2**) alone leads to irreversible enzyme inhibition. The presence of the chloride leaving group of **1** enhances the rate of inhibition, however. We postulate that cross-linking of the active site could be responsible for the potency of **1**.

Materials and Methods

All kinetic measurements were carried out on either a Perkin-Elmer 559 or a Perkin-Elmer λ-3 spectrophotometer in which the cell holder had been maintained at 30.0 ± 0.2 °C by circulating thermostated water. The latter instrument is contained in a Vacuum Atmospheres glovebox under a dry nitrogen atmosphere and was used to carry out rate measurements under anaerobic conditions. Uncorrected melting points were determined with a Mel-Temp apparatus. TLCs were run with Merck silica gel 60 (F_{254}) plates. IR spectra were taken as KBr pellets with a Nicolet MX-1 FTIR spectrophotometer. ^{1}H NMR spectra were taken on a Varian Gemini 300 MHz spectrometer, and chemical shifts are reported relative to TMS. NOE experiments were carried out on a Brucker AM 400 spectrometer. Mass measurements were carried out in the electron-impact mode with a Varian MAT 200 spectrometer. Mass spectral data were used in lieu of elemental analyses. Measurements of pH were made with a Radiometer GK2401C combination electrode. Molecular modeling was carried out with the Polygen Charm and Quanta programs on an IRIS 4080.

Human erythrocyte purine nucleoside phosphorylase, buttermilk xanthine oxidase grade III, guanosine, inosine, and hypoxanthine were purchased from Sigma Chemical Co. The purine nucleoside phosphorylase has a specific activity of 21 units/mg of protein. The salts used in the preparation of buffer solution were of analytical reagent grade obtained from Mallinkrodt and Sigma and were used as such. The buffer solutions were prepared in doubly glass-distilled water.

Reported compounds were prepared as previously described: **1H$_2$** (Lemus & Skibo, 1988), **5** (Baker et al., 1952), **6** (Elderfield et al., 1947), **7** (Baker et al., 1952), **8** (Bogert et al., 1910), **9** (Baker et al., 1952), **14** (Iyer et al., 1956), and **16** (Lemus & Skibo, 1988).

Unreported compounds were prepared as described below.

2-(Chloromethyl)quinazoline-4,5,8(3H)-trione (**1**). To a solution of 21 mg (0.093 mmol) of **1H$_2$** (Lemus & Skibo, 1988) in 0.5 mL of dry methanol, chilled in an ice/salt bath, was added 23 mg (0.10 mmol) of DDQ. The reaction mixture was then stirred for 40 min with continued chilling. The crystalline product was filtered off and washed with generous portions of ethyl acetate: 14-mg (67%) yield; mp 166–172 °C dec; TLC (1-butanol/acetic acid/water [5:3:2]) R_f = 0.59; IR (KBr) 3152, 1725, 1700, 1678, 1570, 1548, 1466, 1106, 858, 663 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 7.02 and 6.88 (2 H, 2 d, J = 10.3 Hz, C(6)H and C(7)H, no assignments made), 4.59 (2 H, s, chloromethylene protons); mass spectrum (EI mode, solids probe) m/z 224 (P$^+$).

8-Amino-7-(methylamino)quinazolin-4(3H)-one (**10**) was prepared by the two-step synthesis described below.

7-(Methylamino)-8-nitroquinazolin-4(3*H*)-one was prepared by heating a stirred mixture of 7-chloro-8-nitroquinazolin-4-(3*H*)-one (Leonard et al., 1975) in 40% aqueous methylamine at 110–120 °C for 15 h. The reaction mixture was then cooled to room temperature, and the crude product was collected and washed with water.

A solution of 500 mg (2.27 mmol) of 7-(methylamino)-8-nitroquinazolin-4(3*H*)-one in 30 mL of 0.5% NaOH solution, containing 100 mg of 5% Pd/C, was shaken under 50 psi of H_2 for 2 h. The completed reaction was filtered through Celite, and the filtrate was neutralized with 5% HCl solution. The crystals that formed were collected and rinsed with water: 326-mg (75%) yield; mp 245–252 °C dec; TLC (1-butanol/acetic acid/water [5:3:2]) R_f = 0.50; IR (KBr) 3356, 3153, 1707, 1619, 1599, 1457, 1319, 1226, 898, 779 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 7.86 (1 H, s, C(2)**H**), 7.38 and 6.70 (2 H, 2 d, J = 8.6 Hz, C(5)H and C(6)H, no assignments made), 5.47 (1 H, m, C(7) amino proton), 4.88 (2 H, s, C(8) amino protons), 2.84 (3 H, d, J = 4.7 Hz, methyl protons). Addition of D_2O resulted in collapse of the C(7) and C(8) amino protons. Mass spectrum (EI mode) m/z 190 (P$^+$).

5,8-Dihydroxyquinazolin-4(3H)-one (**11**) was prepared by the two-step synthesis described below.

A solution of 2.0 g (10.2 mmol) of 2-amino-3,6-dimethoxybenzamide (Skibo, 1985) in 20 mL of 98% formic acid was refluxed for 4 h. The formic acid was evaporated in vacuo, and the residual oil was triturated with ethanol. The resulting product, 5,8-dimethoxyquinazolin-4(3*H*)-one, was filtered and rinsed with ethanol: 1.78-g (85%) yield; mp >230 °C dec (slow darkening); TLC (1-butanol/acetic acid/water [5:3:2]) R_f = 0.51; IR (KBr) 3000, 2837, 2700, 1686, 1573, 1485, 1262, 821, 725, 654, 528, 461 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 11.95 (1 H, br s, N(3)H), 7.90 (1 H, s, C(2)**H**),

7.25 and 6.90 (2 H, 2 d, J = 9.0 Hz, C(6)H and C(7)H, no assignments made), 3.78 and 3.74 (6 H, 2 s, methoxy protons).

The product obtained above, 1.5 g (7.3 mmol), was suspended in 150 mL of dry methylene chloride. To this suspension was added 35.0 mL of 1 M boron tribromide in methylene chloride. The mixture was refluxed for 4 h, after which time the resulting precipitate was filtered and rinsed with methylene chloride. Recrystallization from hot water afforded **11** as white crystals, which were filtered and dried in vacuo: 1.1-g (85%) yield; mp 210–213 °C dec; TLC (1-butanol/acetic acid/water [5:3:2]) R_f = 0.62; IR (KBr) 3326, 3210, 3030, 2907, 1653, 1583, 1467, 1382, 1234, 1031, 836, 653, 620, 534 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 12.52, 11.07, and 9.09 (3 H, 3 br s, N(3)H and hydroxy protons, no assignments made), 8.02 (1 H, s, C(2)H), 7.10 and 6.68 (2 H, 2 d, J = 8.8 Hz, C(6)H and C(7)H, no assignments made).

5,8-Dihydroxy-6-methylquinazolin-4(3H)-one (**12**) was prepared by the four-step synthesis described below.

A solution of 1.5 g (7.7 mmol) of 2,5-dimethoxy-3-methylbenzoic acid (Wessely et al., 1960) in 10 mL of thionyl chloride was refluxed for 1 h. The excess thionyl chloride was evaporated in vacuo, and the residue was dissolved in 30 mL of dry methylene chloride. To this solution was added 30 mL of methylene chloride saturated with ammonia, and the resulting solution was stirred at 25 °C for 10 min. The reaction mixture was evaporated to dryness, and the residue was suspended in 50 mL of water. The aqueous mixture was then extracted with 3 × 50 mL portions of ethyl acetate. The extracts were dried (Na_2SO_4) and were concentrated in vacuo to afford a homogeneous oil. Trituration of the oil with hexane afforded crystalline 2,5-dimethoxy-3-methylbenzamide: 946-mg (63%) yield; mp 115–117 °C; TLC (ethyl acetate/methanol [9:1]) R_f = 0.52; IR (KBr) 3384, 3185, 1640, 1602, 1472, 1398, 1227, 1142, 1063, 1006, 866, 676 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 7.67 and 7.51 (2 H, 2 br s, amide protons), 6.98 and 6.91 (2 H, 2 d, J = 3.3 Hz, aromatic protons, no assignments made), 3.72 and 3.66 (6 H, 2 s, methoxy protons, no assignments made), 2.23 (3 H, s, 3-methyl protons). Addition of D_2O resulted in collapse of the amide protons.

To a solution of 7.0 g (35.9 mmol) of the compound obtained above in 75 mL of dry, distilled acetonitrile, cooled in an ice bath, was added 9.0 g (67.8 mmol) of nitronium tetrafluoroborate portionwise over a 30-min period. Addition of 75 mL of methanol to the completed reaction afforded 2,5-dimethoxy-3-methyl-6-nitrobenzamide as a crystalline solid: 6.2-g (72%) yield; mp 236 °C dec; TLC (ethyl acetate) R_f = 0.43; IR (KBr) 3380, 3173, 1653, 1530, 1478, 1394, 1243, 1093, 1036, 808 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 7.98 and 7.77 (2 H, 2 br s, amide protons), 7.27 (1 H, s, aromatic proton), 3.85 and 3.69 (6 H, 2 s, methoxy protons, no assignments made), 2.31 (3 H, s, methyl protons). Addition of D_2O resulted in collapse of the amide protons.

A mixture consisting of 4.0 g (16.7 mmol) of the product obtained above and 300 mg of 5% Pd/C in 150 mL of methanol was shaken under 40 psi of H_2 for 5 h. The completed reaction was filtered through Celite and the filtrate was evaporated to dryness. The residue was dissolved in 50 mL of 96% formic acid and refluxed for 10 h. The formic acid was evaporated in vacuo, and the product, 5,8-dimethoxy-6-methylquinazolin-4(3H)-one, was recrystallized from methanol: 3.2-g (87%) yield; mp 244–255 °C dec; TLC (ethyl acetate/acetic acid [9:1]) R_f = 0.26; IR (KBr) 3060, 2928, 1678, 1609, 1480, 1333, 1235, 1192, 1062, 953 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 11.94 (1 H, br s, N(3)H), 7.91 and 7.24 (2 H, 2 s, C(2)H and C(7)H, no assignments made), 3.84 and 3.68 (6 H, 2 s, methoxy protons, no assignments made), 2.31 (3 H, s, C(6) methyl protons).

To a suspension of 500 mg (2.3 mmol) of the product obtained above in 10 mL of dry benzene was added 0.5 mL of 99% BBr_3, and the resulting mixture was stirred at reflux for 3 h. The completed reaction was cooled to room temperature and treated with 5.0 mL of ethanol. Evaporation of the ethanolic solution in vacuo afforded an oil, which crystallized upon addition of acetone: 263-mg (60%) yield; TLC (1-butanol/acetic acid/water [5:3:2]) R_f = 0.67; IR (KBr) 3333, 3160, 3016, 2919, 1647, 1577, 1467, 1397, 1222, 998, 658, 545, 429 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 11.22 (1 H, br s, N(3)H), 8.19 and 7.12 (2 H, 2 s, C(2)H and C(7)H, no assignments made), 2.18 (3 H, s, C(6) methyl protons); mass spectrum (EI mode) m/z 192 (P^+).

5-Hydroxyquinazolin-4(3H)-one (**13**). To a suspension of 150 mg (0.85 mmol) of 5-methoxyquinazolin-4(3H)-one (Baker & Shaub, 1954) in 20 mL of dry methylene chloride was added 2.0 mL of 1 M BBr_3 in methylene chloride. The mixture was refluxed for 3 h, and the precipitated solid was filtered off and recrystallized from water: 86-mg (62%) yield; mp >205 °C dec (slow darkening); TLC (ethyl acetate/acetic acid [9:1]) R_f = 0.43; IR (KBr) 2817, 2666, 1670, 1635, 1611, 1463, 1259, 1250, 1064, 823, 770 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 8.14 (1 H, s, C(2)H), 7.69 (1 H, t, J = 8.2 Hz, C(7)H), 7.12 and 6.87 (2 H, 2 d, J = 8.3 Hz, C(6)H and C(8)H, no assignments made).

8-Amino-5-hydroxyquinazolin-4(3H)-one hydrobromide (**15**·*HBr*) was prepared by the three-step synthesis described below.

To a suspension of 500 mg (2.8 mmol) of 5-methoxyquinazolin-4(3H)-one (Baker & Schaub, 1954) in 10 mL of dry acetonitrile at 0 °C was added 575 mg (4.3 mmol) of nitronium tetrafluoroborate portionwise over a 5-min period. The reaction mixture was stirred for an additional 10 min, and the crystals that formed (pure 5-methoxy-8-nitroquinazolin-4(3H)-one) were filtered and rinsed with ethyl acetate: 234-mg (37%) yield. The filtrate, containing both 6- and 8-nitro isomers, was evaporated to dryness, and the residue was recrystallized from acetic acid to afford an 87-mg (14%) yield of additional pure 8-nitro isomer. Physical properties of the 8-nitro isomer: mp 212–218 °C dec; TLC (ethyl acetate/acetic acid [9:1]) R_f = 0.26; IR (KBr) 3541, 3176, 3070, 2894, 1660, 1605, 1522, 1355, 1268, 1071, 840 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 8.24 (1 H, d, J = 9.0 Hz, C(7)H), 8.15 (1 H, s, C(2)H), 7.13 (1 H, d, J = 9.1 Hz, C(6)H), 3.96 (3 H, s, methoxy protons). The assignment for the C(6) proton was based on a strong NOE effect observed with the C(5) methoxy protons. No NOE effect was observed between the C(7) proton and the C(5) methoxy protons. Mass spectrum (EI mode) m/z 221 (P^+).

A mixture consisting of 200 mg (0.90 mmol) of the product obtained above and 50 mg of 5% Pd/C in 30 mL of methanol was shaken under 50 psi of H_2 for 1 h. The completed reaction was filtered through Celite and was evaporated to dryness. The residue, 8-amino-5-methoxyquinazolin-4(3H)-one, was recrystallized from methanol/ethyl acetate: 143-mg (83%) yield; mp 205–220 °C dec; TLC (2-propanol/water/ammonium hydroxide [7:2:1]) R_f = 0.70; IR (KBr) 3311, 3144, 2954, 1672, 1629, 1577, 1474, 1272, 1074, 826, 516, 461 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 7.90 (1 H, s, C(2)H), 6.96 and 6.82 (2 H, 2 d, J = 8.6 Hz, C(6)H and C(7)H, no assignments made), 5.12 (2 H, br s, 8-amino protons), 3.71 (3 H, s, methoxy protons). Addition of D_2O resulted in collapse

of the 8-amino protons; mass spectrum (EI mode) m/z 191 (P^+).

A solution of 430 mg (2.3 mmol) of the product obtained above in 8.0 mL of 48% aqueous hydrobromic acid was stirred at reflux for 12 h. The crystals (**15**·HBr) that formed were collected and rinsed with absolute ethanol: 302-mg (52%) yield; mp >250 °C dec (slow darkening); TLC (2-propanol/water/ammonium hydroxide [7:2:1]) R_f = 0.73; IR (KBr) 3037, 2852, 1664, 1634, 1491, 1229, 1037, 814, 662, 464 cm^{-1}; ^1H NMR (dimethyl-d_6 sulfoxide) δ 8.33 (1 H, s, C(2)H), 7.71 and 6.94 (2 H, 2 d, J = 8.8 Hz, C(6)H and C(7)H, no assignments made); mass spectrum (EI mode) m/z 177 (P^+).

The base form of **15**·HBr was obtained by dissolving 100 mg (3.9 mmol) of the salt in 0.2 M potassium phosphate buffer (2.0 mL), pH 7.0. The crystals that formed were collected and rinsed with water: 60-mg (87%) yield; ^1H NMR (dimethyl-d_6 sulfoxide) δ 12.4 (1 H, br s, N(3)H), 10.84 (1 H, s, phenol proton), 8.00 (1 H, s, C(2)H), 7.01 and 6.67 (2 H, 2 d, J = 8.6 Hz, C(6)H and C(7)H, no assignments made), 5.02 (2 H, br s, 8-amino protons). Addition of D_2O resulted in collapse of the N(3), phenol, and 8-amino protons.

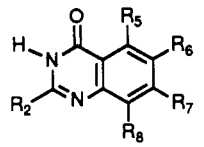
6-Methylquinazoline-4,5,8(3H)-trione (**17**). To a suspension of 50 mg (0.23 mmol) of 5,8-dimethoxy-6-methylquinazolin-4(3*H*)-one (see third step under **12**) in 3.0 mL of water was added 300 mg (0.55 mmol) of ceric ammonium nitrate. The mixture was stirred for 30 min at room temperature. The yellow crystals that formed were collected and rinsed with water: 35-mg (80%) yield; mp >215 °C dec (slow darkening); TLC (1-butanol/acetic acid/water [5:3:2]) R_f = 0.55; IR (KBr) 3581, 3519, 2856, 1710, 1670, 1540, 1428, 1328, 1227, 1133, 1106, 831 cm^{-1}; ^1H NMR (dimethyl-d_6 sulfoxide) δ 8.53 and 6.91 (2 H, s and d, respectively, J = 1.6 Hz, C(7)H and C(2)H, respectively, doublet result of C(2)H splitting by N(3)H), 2.02 (3 H, s, C(6) methyl protons); mass spectrum (EI, solids probe), m/z 192 (P^+ + 2), 190 (P^+). The P^+ + 2 mass corresponds to hydroquinone formed on the solids probe.

Quinazoline-4,5,8(3H)-trione (**18**). To a suspension of 200 mg (1.1 mmol) of **11** in 15 mL of dry methanol was added 500 mg (2.2 mmol) of dichlorodicyanobenzoquinone (DDQ). The mixture was stirred for 1 h at 25 °C, and the resulting yellow solid was filtered and recrystallized from 95% ethanol: 96-mg (49%) yield; mp >320 °C dec; TLC (1-butanol/acetic acid/water, [5:3:2]) R_f = 0.58, IR (KBr) 3571, 3040, 2837, 1711, 1677, 1612, 1542, 1425, 1308 cm^{-1}; ^1H NMR (dimethyl-d_6 sulfoxide) δ 8.55 (1 H, s, C(2)H), 7.01 and 6.87 (2 H, 2 d, J = 10.4 Hz, C(6)H and C(7)H, no assignments made); mass spectrum (EI mode, solids probe) m/z 178 (P^+ + 2), 176 (P^+). The P^+ + 2 mass corresponds to hydroquinone formed on solids probe.

Phosphate-Dependence Studies. PNPase activity (inosine to hypoxanthine and ribose 1-phosphate) was followed by measuring the increase in absorbance at 292 nm accompanying the oxidation of hypoxanthine to uric acid by xanthine oxidase; $\Delta\epsilon$ value for the hypoxanthine to uric acid conversion is 1.29 × 10^4 M^{-1} cm^{-1} (Dempcy & Skibo, 1991).

Reaction mixtures for inhibition studies of **5** and **6** contained 0.2 M pH 7.50 Tris buffer (μ = 1.0 KCl) with various fixed concentrations of potassium phosphate ranging from 10.0 to 200 mM; xanthine oxidase, 0.06 unit; PNPase, 0.005 unit; and inosine concentrations ranging from 0.020 to 0.50 mM. Stock solutions of the inhibitors were prepared in dimethyl sulfoxide. At each constant phosphate concentration, 2–4 K_i values were determined for each of the inhibitors and the average values were plotted on Figure 2.

Table I: K_i Values for PNPase Inhibition by Substituted Quinazolin-4(3*H*)-ones



no.	R_2	R_8	R_7	R_6	R_5	K_i (μM)
7	H	H	NH_2	H	H	NA
8	H	H	H	NH_2	H	NA
9	H	H	H	H	NH_2	337
10	H	NH_2	$NHCH_3$	H	H	NA
11	H	OH	H	H	OH	142
12	H	OH	H	CH_3	OH	618
13	H	H	H	H	OH	576
14	H	OH	H	H	H	593
15	H	NH_2	H	H	OH	131

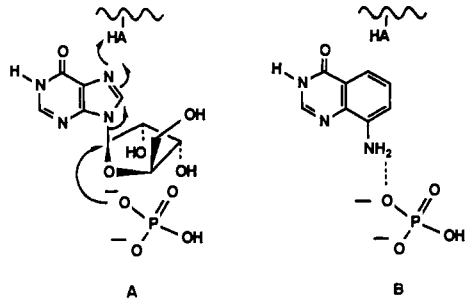
Reaction mixtures for the PNPase negative cooperativity study, Figure 1, contained 0.2 M Tris buffer, pH 7.50 (μ = 1.0 KCl), with concentrations of potassium phosphate ranging from 10.0 to 582 mM; xanthine oxidase, 0.06 unit; PNPase, 0.005 unit; and inosine, 0.040 mM.

Competitive Inhibition Studies. PNPase activity (guanosine to guanine and ribose 1-phosphate) was followed by measuring the decrease in absorbance at 268 nm using a $\Delta\epsilon$ value of 2.38 × 10^3 M^{-1} cm^{-1}. By assaying PNPase activity in this fashion, rather than by the coupled xanthine oxidase assay, it was possible to determine K_i values under anaerobic conditions. Caveat: Turbulence causes rapid loss of activity when enzyme solutions are deoxygenated by bubbling in argon or nitrogen. Anaerobic solutions of enzyme were prepared by vacuum degassing of solid PNPase followed by dissolution in degassed buffer.

Reaction mixtures contained 0.2 M Tris buffer, pH 7.4 (μ = 1.0 KCl), PNPase (0.008–0.01 unit), and guanosine concentrations ranging from 0.02 to 0.13 mM. Stock solutions of inhibitors were prepared in dimethyl sulfoxide. The unit activity of nucleoside phosphorylase from human blood (Sigma) was determined spectrophotometrically. Assays of inhibitors were usually carried out under aerobic conditions except for the hydroquinone inhibitors, which were assayed under anaerobic conditions. K_i values from these assays are summarized in Table I.

Time-Dependent Irreversible Inactivation of PNPase. PNPase activity (guanosine to guanine and ribose 1-phosphate) was followed by measuring the decrease in absorbance at 268 nm using a $\Delta\epsilon$ value of 2.38 × 10^3 M^{-1} cm^{-1}. Incubation mixtures contained 0.05 M potassium phosphate buffer, pH 7.40 (μ = 0.10 KCl); PNPase, 0.95 unit; and 0.368 mM of inhibitors **1**, **16**, and $\mathbf{1H_2}$. The quinone inhibitors (**1** and **16**) were assyed under aerobic and anaerobic conditions, and the hydroquinone ($\mathbf{1H_2}$) was assayed under anaerobic conditions. Aliquots of the incubation mixtures were assayed at various time intervals by mixing with buffer containing 0.067 mM guanosine. Activity vs time data (not shown) were fit to a first-order rate law to provide the k_{obsd} values for irreversible inactivation. The k_{obsd} values were then determined at various inhibitor concentrations, and the apparent second-order rate constants for inactivation were obtained from k_{obsd} vs [inhibitor] plots (not shown). The study of the influence of hypoxanthine on rate of inactivation (Figure 4) was carried out in incubation mixtures, prepared as described above, but with added hypoxanthine. At various time intervals, aliquots were assayed by dilution with buffer containing 0.067 mM guanosine.

Chart I



Titration of PNPase with **1**. Plots of V_{max} for phosphorolysis vs [PNPase] were obtained in the presence and absence of inhibitor [see Segel (1975) for examples of these plots]. The extrapolated x intercept provided the concentration of enzyme inactivated by a given concentration of inhibitor and thereby provided the inactivation ratio [inhibitor]/[PNPase].

PNPase activity was measured by following guanosine phosphorolysis at 268 nm. Incubation mixtures contained 0.05 M potassium phosphate buffer, pH 7.40 (μ = 0.1 KCl), PNPase concentrations ranging from 3.9 to 20 nM, and 7.4 μM inhibitor. The incubation mixtures were assayed by dilution of aliquots with buffer containing guanosine. Values for maximum velocity were obtained from the extrapolated y intercept of reciprocal plots of velocity vs guanosine concentration. The approximate molarity of enzyme was calculated directly from the weight of enzyme employed. This value was confirmed by titration of the enzyme stock solution with 5,5′-dithiobis(2-nitrobenzoic acid) with use of a $\Delta\epsilon$ value of 13 000 at 412 nm and the assumption that on average 4.5 mol of DTNB will react with 1 mol of PNPase (Agarwal & Parks, 1971).

Enzyme Isolation Study. PNPase (0.20 mg), dissolved in 1.5 mL of 0.05 M potassium phosphate buffer, pH 7.4 (μ = 0.10 KCl), was incubated with 3.0 mg of $\mathbf{1H_2}$ for 12 h at 30 °C under anaerobic conditions. An aliquot was assayed for guanosine phosphorolysis at this time, and no PNPase activity was observed. The incubation mixture was chromatographed through a Sephadex column (G-25, L = 19.0 cm, w = 1.2 cm) and eluted with water. The enzyme fractions were monitored by a UV detector at 280 nm. The enzyme fractions were pooled and once again chromatographed through a fresh column. The isolated enzyme showed no activity toward guanosine phosphorolysis. The UV spectrum of the isolated enzyme showed λ_{max} = 360 nm (major) and λ_{max} = 410 nm (minor) not present in the native enzyme.

In a control study (no inhibitor present), the chromatographed enzyme still catalyzed guanosine phosphorolysis.

Results and Discussion

PNPase Active-Site Tolerance for Quinazolines. The purine-like properties of the quinazoline ring system permitted its use in the design of a xanthine oxidase reductive alkylating agent (Lemus & Skibo, 1988). The strategy for designing a PNPase reductive alkylating agent thus involved functionalizing the quinazoline ring as a quinone with an appropriately placed leaving group (e.g., **1** in Scheme I). A nucleophilic residue group in the PNPase active site could reductively add to the quinone resulting in the hydroquinone (**1** → **2**). Elimination of the leaving group from the hydroquinone could then afford an alkylating quinone methide species (**2** → **3**), which could alkylate another nucleophilic residue. These reactions would afford a cross-linked PNPase active site by a process initiated by reductive addition. The success of this inhibitor design depends on how the quinazoline ring binds in the PNPase active site as well as the locations of nucleophiles therein. The first steps in the rational design were therefore to define the position of the active-site-bound quinazoline ring relative to catalytic residues and to define the tolerance of the active site for substituted quinazolines.

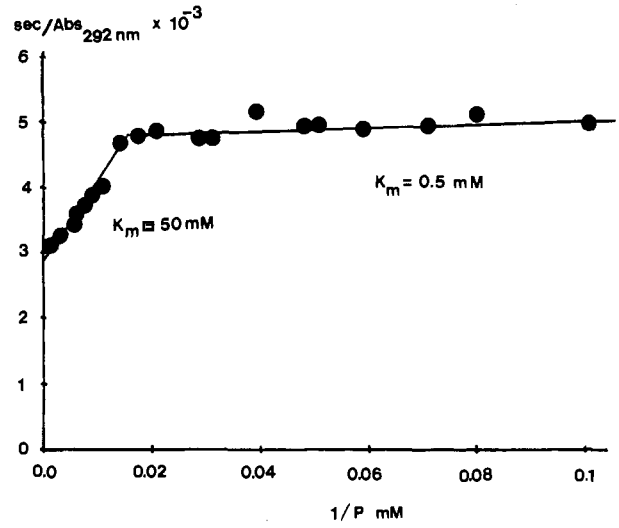


FIGURE 1: Reciprocal plot for inosine (0.04 mM) phosphorolysis by PNPase wherein phosphate is the variable substrate (10–582 mM). The rate of phosphorolysis was followed by utilizing the xanthine oxidase coupled assay.

In a recent paper (Dempcy & Skibo, 1991), we mentioned that quinazolin-4(3*H*)-one (**5**) and the 8-amino analogue (**6**) are both competitive inhibitors of PNPase. The 8-amino group of **6** was proposed to interact with enzyme-bound phosphate by means of hydrogen bonding or a salt interaction (structure B in Chart I). Indeed, superimposition of computer energy-minimized structures of **6** and of inosine places the 8-amino group at the site where phosphate attack occurs (structure A in Chart I). Verification of an interaction would thus serve to define the mode of quinazoline binding in the PNPase active site.

The negative cooperativity for phosphate binding to the PNPase active site assisted in confirming an interaction between **6** and enzyme-bound phosphate. PNPase from various sources exists as a trimer (Agarwal et al., 1975), and the binding of each phosphate to a subunit decreases the affinity of vacant subunits for phosphate (negative cooperativity). For example, bovine thyroid PNPase displays a downward curving reciprocal plot when phosphate is the variable substrate due to the change in the phosphate dissociation constant (Moyer et al., 1980). As shown in Figure 1, human erythrocyte PNPase also exhibits a downward curving reciprocal plot. From the data in Figure 1, we were able to determine the phosphate concentrations where an additional phosphate is added to the trimer: $EP_{n+1} \rightleftarrows EP_n + P$, where the K_m for phosphate dissociation from EP_n = 0.5 mM and K_m for phosphate dissociation from EP_{n+1} is 50 mM. If **6** interacts with enzyme-bound phosphate, the K_i of **6** will be dependent on the K_m for phosphate.

The data in Figure 2 support an interaction between **6** and enzyme-bound phosphate. At phosphate concentrations where the K_m of enzyme-bound phosphate is 50 mM, the K_i for **6** is 80 μM. At low phosphate concentrations, where the phosphate K_m = 0.5 mM, the K_i for **6** is ~10 μM. In the absence of an 8-amino group, as in **5**, the K_i of the inhibitor is much higher than those of **6** and is independent of phosphate concentration (Figure 2).

The results presented thus far indicate that quinazolines bind

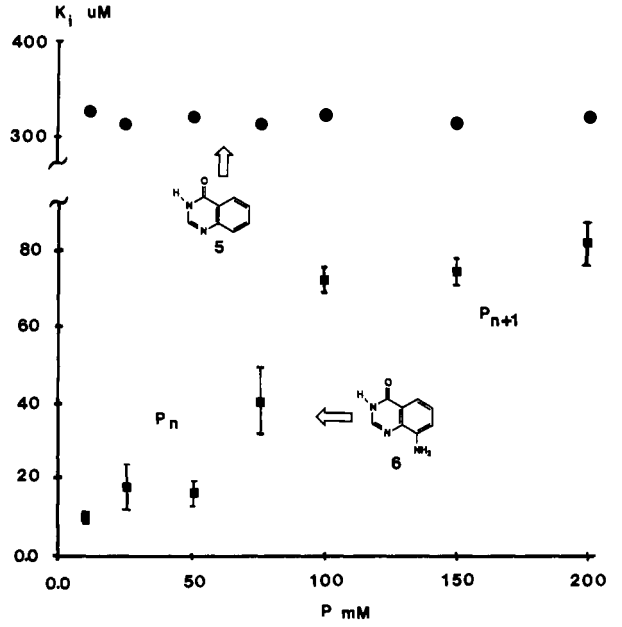


FIGURE 2: Plot of K_i vs phosphate concentration for two PNPase inhibitors, **5** and **6**. Each K_i value is the average of two to four K_i determinations. The K_i values were obtained from Lineweaver–Burk plots.

to the PNPase active site in a fashion similar to that of purine substrates. Additional evidence for the purine-like behavior of quinazolines is the influence of substituents on binding. In a previous publication, we showed that substitution of a 2-hydroxyl group on **6** diminishes binding while the substitution of a 2-amino group on **6** retains binding (Dempcy & Skibo, 1991). This behavior is analogous to xanthine and guanine binding to the PNPase active site, respectively. In the present study, substitutions were made on the fused benzene ring of quinazolin-4(3*H*)-one, and the tolerance of the active site for these substituents were determined by measuring K_i values (Table I). Substitution of amino groups on the 6- and 7-positions result in loss of competitive inhibition (**7**, **8**, and **10**), but the active site tolerates a methyl at the 6-position (**12**). The active site also tolerates substitution at the 5- and/or 8-positions (**11**, **12**, **13**, and **15**). These findings parallel the binding behavior of substituted purines to the human erythrocyte PNPase active site. Bulky substituents at the 8-position of purine substrates result in loss of binding affinity (Jordan & Wu, 1978). In superimposed models, the 8-position of the purine ring is located near the 6- and 7-positions of the quinazoline ring. However, the 6- and 7-positions are slightly extended beyond the 8-position of the purine ring.

The results enumerated above suggest that quinazoline analogues bind like purine substrates to the PNPase active site. Although the quinazolines are weak competitive inhibitors of PNPase, these systems can still act as vehicles to deliver alkylating centers to active-site nucleophiles.

Nucleophile Search. The next step in the rational inhibitor design was to search the PNPase active site for nucleophiles. Since the quinazoline ring system binds to the PNPase active site like a purine substrate (see above), a docking study was carried out wherein the pyrimidine ring of **1** was superimposed on the pyrimidine ring of substrate bound to the three-dimensional PNPase structure (Ealick et al., 1990). The results of this docking study are shown in Figure 3. Glutamate 201, which interacts with the 1-position of the purine substrate, is in position to react with the 2α alkylating center of **1**. Glutamate 259 is in position to undergo reductive addition at the 6-position of **1**. Initially, it was proposed that an active-site

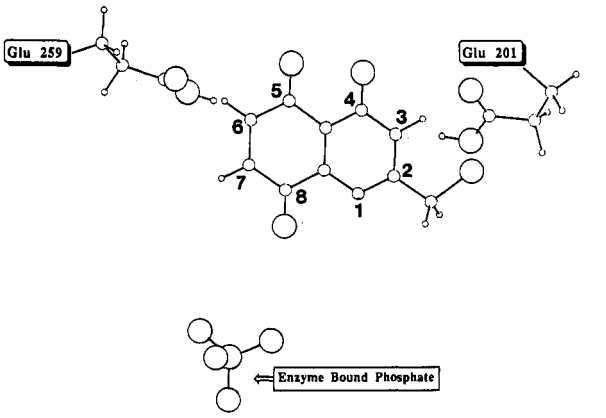
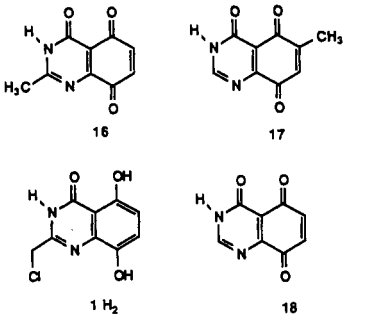


FIGURE 3: The relative orientation of **1** to the glutamate 201 and 259 residues and to enzyme-bound phosphate in the PNPase active site.

Chart II



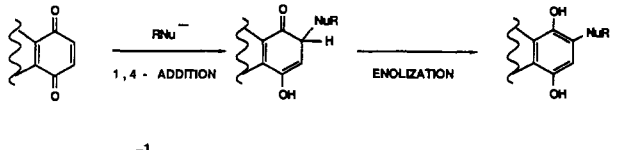
sulfhydryl residue (Carlson & Fisher, 1979) could reductively add to **1**. Actually, a sulfhydryl residue is only located *near* the active site (Ealick et al., 1990) and the docking study showed that this residue is too far from the 6-position of **1**.

The nucleophile search involved the screening of **1**, reduced **1** ($\mathbf{1H_2}$), and the quinones **16**, **17**, and **18** in Chart II as irreversible inhibitors of PNPase. Quinones **16** and **18** inactivate the enzyme presumably by reductive addition of Glu 259. The quinone that cannot undergo reductive addition (**17**) does not inactivate the enzyme. Reduced **1** ($\mathbf{1H_2}$) inactivates the enzyme, presumably by alkylation of Glu 201. Finally, **1** appears to inactivate the enzyme by cross-linking Glu 201 and Glu 259.

Compounds **1**, $\mathbf{1H_2}$, **16**, and **18** inactivate PNPase by time-dependent processes. PNPase activity was determined as a function of time, and the data were fit to a first-order rate law, which was used to generate the solid curves (not shown). First-order rate constants, obtained over a range of inhibitor concentrations, were plotted against inhibitor concentration. The slopes of these lines are the apparent second-order rate constants for PNPase inhibition: **1** (222.3 M^{-1} min^{-1}), **16** (97.4 M^{-1} min^{-1}), and $\mathbf{1H_2}$ (29.6 M^{-1} min^{-1}). The inactivation kinetics of **18** are the same as those of **16**. The linear plots (observed first-order rate constants vs inhibitor concentration) indicate that saturation in inhibitor is never achieved (the curves would approach a slope of 0 at high inhibitor concentrations if saturation were occurring). Inhibitors **1** and **16** show identical inactivation kinetics in aerobic and anaerobic buffers. Compound $\mathbf{1H_2}$ was only studied in anaerobic buffer due to the oxygen-mediated oxidation of $\mathbf{1H_2}$ to **1**.

Discussions of the mechanisms of inhibition by the quinones (**1** and **16**) and the hydroquinone $\mathbf{1H_2}$ follow. The quinones **1** and **16** are proposed to inactivate PNPase by a mechanism involving reductive addition of Glu 259 at the 6-position. Reductive addition is a two-step process consisting of 1,4 addition of a nucleophile to the quinone ring followed by enolization of the intermediate to the hydroquinone (Scheme

Scheme II



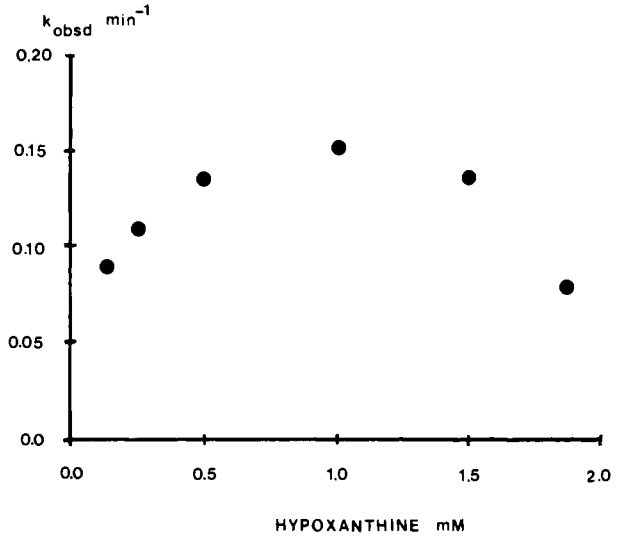


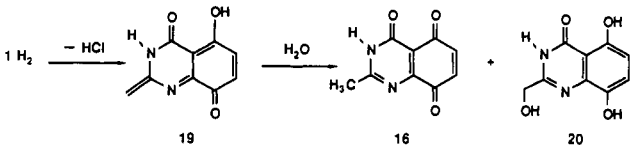
FIGURE 4: Plot of k_{obsd} vs hypoxanthine concentration for the inactivation of 0.95 unit of PNPase by 0.368 mM of **1** in 0.05 M phosphate buffer, pH 7.4 (μ = 0.1 KCl), at 30 °C and at various concentrations of hypoxanthine.

II). If enzyme inactivation involves nucleophile addition at the 6-position of the quinazoline quinone, the 6-methyl derivative (**17**) should not inactivate the enzyme since enolization cannot occur. In accord with nucleophile addition at the 6-position, **17** does not inactivate PNPase. It should be pointed out that the 6-methyl substituent does not hamper entry into the active site (see **12** in Table I), and thus exclusion of **17** from the active site is not an explanation for the lack of inactivation.

Nucleophile addition reactions, other than reductive addition of the 6-position, are dismissed as possibilities with the following arguments. Nucleophile addition to the 5-carbonyl of **1**, **16**, and **18** cannot occur due to the delocalized negative charge arising from dissociation of the N(3) proton, pK_a ~ 6 (Skibo, 1985). Indeed, a study of the nucleophile-addition chemistry of quinazoline quinones revealed that addition reactions only occur at the 6- and 8-carbon centers (Skibo, 1985). Finally, if nucleophile addition to the 7- or 8-positions was important, the 6-methyl analogue (**17**) would be active.

Data consistent with a quinone interaction at the PNPase active site were obtained by studying the effect of added hypoxanthine on the rate constant for PNPase inactivation by **16**. The plot of k_{obsd} for PNPase inactivation vs [hypoxanthine] is shown in Figure 4. This bell-shaped plot indicates that hypoxanthine has the effect of both promoting and inhibiting enzyme inactivation. This duality of effects could be explained by substrate activation (negative cooperativity) and by hypoxanthine-mediated exclusion of the inhibitor from the active site. PNPase from all sources displays apparent substrate activation, which may be the result of cooperativity among the subunits of the enzyme (Agarwal et al., 1975). This cooperativity may be due to a conformational change in the enzyme upon substrate binding to a subunit, resulting in altered kinetic properties of the vacant subunits. The binding of hypoxanthine to a subunit could thus activate the vacant subunits of PNPase toward inactivation by **16** by altering the positions of active-site catalytic residues. The reciprocal replot

Scheme III



of the plot in Figure 4 (not shown) provides, as the reciprocal of the *y* intercept, the maximal rate constant for inactivation in the presence of hypoxanthine (0.16 min^{-1}). At high hypoxanthine concentrations, hypoxanthine binding to all three subunits would be favored competitively and the inhibitor would be excluded from the active sites. The descending curve at high hypoxanthine concentrations indicates active-site protection from the inhibitor.

Quinone **1** is the best PNPase inhibitor in terms of the rate constant for enzyme inactivation: 222.3 M^{-1} min^{-1} for **1** vs 97.4 M^{-1} min^{-1} for **16**. Inductive electron withdrawal by the 2-(chloromethyl) group was considered as an explanation for the activity of **1**. Addition of the glutamate nucleophile to an electron-deficient quinone would be expected to occur with facility. However, the identical inactivation rates of **16** and **18** suggest that the electronic character of the 2-substituent is not important in this respect. The chemistry of reduced **1** [see Scheme III and Lemus and Skibo (1988)] suggests that a quinone methide species would be formed in the active site upon reduction. Our studies (Lemus & Skibo, 1988) show that the quinone methide species derived from $\mathbf{1H_2}$ (**19** in Scheme III) is capable of trapping an oxygen nucleophile (water and hydroxide). The proximity of a glutamate residue to the 2-position of enzyme-bound inhibitor (Figure 3) suggests that alkylation of this residue could occur. In fact, $\mathbf{1H_2}$ inactivates PNPase by a first-order process (loc. cit.). We propose that cross-linking of the active site by **1** accounts for the facility of enzyme inactivation. The presence of only one cross-linking event could inactivate the PNPase trimer due to conformational changes. On the other hand, monoalkylation of all three subunits of PNPase by **16** or **18** may be necessary for complete inactivation.

Stoichiometry and Characterization of Inhibited Enzyme. The minimum [**1**]/[PNPase] ratio needed for complete enzyme inactivation by **1** had measured values from 150 up to 1250. The value of the ratio depends on the amount of **1** present in incubation mixtures and the incubation time. The first-order inactivation of PNPase is dependent on the concentration of **1**. A low concentration of **1** will therefore require up to a day to inactivate the enzyme, but the [**1**]/[PNPase] ratio for complete inactivation will be low. On the other hand, increasing the concentration of **1** decreases the inactivation time and accordingly increases the value of this ratio. The ideal 1/1 stoichiometry may not be achievable, since the incubation times will extend well beyond the half-life of **1** hydrolysis to inactive species.

Evidence that inactivation is irreversible was obtained from purification of inactivated bulk enzyme on a Sephadex column (see Materials and Methods). Column purification of inactivated enzyme did not result in a return of guanosine phosphorolysis activity, while this activity in native enzyme was unaffected by column purification. Furthermore, the UV–visible spectrum of purified, inactivated enzyme showed additional λ_{max} values not present in the native enzyme (λ_{max} values = 360 and 410 nm). Indeed these are typical λ_{max} values of a quinazoline quinone (Lemus & Skibo, 1988). The enzyme hydroquinone adducts proposed in Scheme I, **2** and **4**, could air oxidize to afford the corresponding quinone adducts.

CONCLUSIONS

Our results show that the quinazolin-4(3*H*)-one ring system will enter the active site of human erythrocyte PNPase in much the same fashion as purine substrates. The implication is that new inhibitors of PNPase can be designed by appropriate functionization of the quinazoline ring.

In the present study, we show that a reactive benzoquinone moiety can be introduced into the active site by utilizing quinazoline quinone derivatives (**1**, **16**, and **18**). Enzyme inactivation may pertain to glutamate addition to the 6-position to afford an active-site-bound hydroquinone. In the case of **1**, this hydroquinone may eliminate a leaving group to afford a quinone methide species capable of alkylating another active-site glutamate residue.

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REFERENCES

Agarwal, R. P., & Parks, R. E., Jr. (1971) *J. Biol. Chem. 246*, 3763–3768.

Agarwal, K. C., Agarwal, R. P., Stoeckler, J. D., & Parks, R. E., Jr. (1975) *Biochemistry 14*, 79–84.

Baker, B. R., & Schaub, R. E. (1954) British Patent 713767, CA 50: P14002, 1956.

Baker, B. R., Schaub, R. E., Joseph, J. P., McEvoy, F. J., & Williams, J. H. (1952) *J. Org. Chem. 17*, 141–148.

Bogert, M. T. Beal, G. D., & Amend, C. G. (1910) *J. Am. Chem. 32*, 1654–1665.

Carlson, J. D., & Fischer, A. G. (1979) *Biochim. Biophys. Acta 571*, 21–34.

Daddona, P. E., Wiesmann, W. P., Milhouse, W., Chern, J.-W., Townsend, L. B., Hershfield, M. S., & Webster, H. K. (1986) *J. Biol. Chem. 261*, 11667–11673.

Dempcy, R. O., & Skibo, E. B. (1991) *J. Org. Chem. 56*, 776–785.

Ealick, S. E., Rule, S. A., Carter, D. C., Greenhough, T. J., Babu, Y. S., Cook, W. J., Habash, J., Helliwell, J. R., Stoeckler, J. D., Parks, R. E., Jr., Chen, S., & Bugg, C. E. (1990) *J. Biol. Chem. 265*, 1812–1820.

Finley, K. T. (1974) in *The Chemistry of the Quinonoid Compounds* (Patai, S., Ed.) Part 2, pp 877–1144, John Wiley, New York (see pp 882–898 for a discussion of sulfur addition reactions to quinones).

Iyer, R. N., Anand, N., & Dhar, M. L. (1956) *J. Sci. Ind. Res. 15C*, 1–7.

Jordan, F., & Wu, A. (1978) *J. Med. Chem. 21*, 877–882.

Kazmers, I. S., Mitchell, B. S., Dadonna, P. E., Wotring, L. L., Townsend, L. B., & Kelley, W. N. (1981) *Science 214*, 1137–1139.

Krenitsky, T. A., Tuttle, J. V., Miller, W. H., Moormann, A. R., Orr, G. F., & Beauchamp, L. (1990) *J. Biol. Chem. 265*, 3066–3069.

Lemus, R. H., & Skibo, E. B. (1988) *J. Org. Chem. 53*, 6099–6105.

Leonard, N. J., Morrice, A. G., & Sprecker, M. A. (1975) *J. Org. Chem. 40*, 356–363.

Moyer, T. P., Fischer, A. G., & Schulz, A. R. (1980) *Int. J. Biochem. 11*, 243–250.

Nakamura, C. E., Chu, S.-H., Stoeckler, J. D., & Parks, R. E., Jr. (1986) *Biochem. Pharmacol. 35*, 133–136.

Parks, R. E., Jr., & Agarwal, R. P. (1972) *Enzymes (3rd Ed.) 7*, 483–514.

Parks, R. E., Jr., Stoeckler, J. D., Cambor, C., Savarese, T. M., Crabtree, G. W., & Chu, S.-H. (1981) in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Sartorelli, A., Lazo, J. S., & Bertino, J. R., Eds.) pp 229–252, Academic Press, New York.

Salamone, S. J., & Jordan, F. (1982) *Biochemistry 21*, 6383–6388.

Segel, I. H. (1975) *Enzyme Kinetics*, p 128, Wiley, New York.

Shewach, D. S., Chern, J.-W., Pillote, K. E., Townsend, L. B., & Daddona, P. E. (1986) *Cancer Res. 46*, 519–523.

Sircar, J. C., Suto, M. J., Scott, M. E., Dong, M. K., & Gilbertsen, R. B. (1986) *J. Med. Chem. 29*, 1804–1806.

Skibo, E. B. (1985) *J. Org. Chem. 50*, 4861–4865.

Stein, J. M., Stoeckler, J. D., Li, S.-Y., Tolman, R. L., MacCoss, M., Chen, A., Karkas, J. D., Ashton, W. T., & Parks, R. E., Jr. (1987) *Biochem. Pharmacol. 36*, 1237–1244.

Stoeckler, J. D., Cambor, C., Kuhns, V., & Chu, S.-H. (1982) *Biochem. Pharmacol. 31*, 163–171.

Stoeckler, J. D., Ealick, S. E., Bugg, C. E., & Parks, R. E., Jr. (1986a) *Fed. Proc. 45*, 2773–2778.

Stoeckler, J. D., Ryden, J. B., Parks, R. E., Jr., Chu, M.-Y., Lim, M.-I., Ren, W. Y., & Klein, R. S. (1986b) *Cancer Res. 46*, 1774–1778.

Wessely, F., Zbiral, E., & Sturm, H. (1960) *Chem. Ber. 93*, 2840–2851.