Rögner, M., Nixon, P. J., & Diner, B. A. (1990) *J. Biol. Chem. 265*, 6189–6196.
Rutherford, A. W. (1989) *Trends Biochem. Sci. 14*, 227–232.
Rutherford, A. W., & Mathis, P. (1983) *FEBS Lett. 154*, 328–334.
Rutherford, A. W., & Zimmermann, J.-L. (1984) *Biochim. Biophys. Acta 767*, 168–175.
Rutherford, A. W., Paterson, D. R., & Mullet, J. E. (1981) *Biochim. Biophys. Acta 635*, 205–214.
Rutherford, A. W., Crofts, A. R., & Inoue, Y. (1982) *Biochim. Biophys. Acta 682*, 457–465.
Rutherford, A. W., Zimmermann, J.-L., & Mathis, P. (1984) *FEBS Lett. 165*, 156–162.
Rutherford, A. W., Boussac, A., & Zimmermann, J.-L. (1991) *New J. Chem. 15*, 491–500.
Rutherford, A. W., Zimmermann, J.-L., & Boussac, A. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) Elsevier, London (in press).
Sivaraja, M., Tso, J., & Dismukes, G. C. (1989) *Biochemistry 28*, 9459–9464.
Stewart, A. C., Ljungberg, V., Åkerlund, H.-E., & Andersson, B. (1985) *Biochim. Biophys. Acta 808*, 353–362.
Styring, S., & Rutherford, A. W. (1987) *Biochemistry 26*, 2401–2405.
Van Mieghem, F. J. E., Nitschke, W., Mathis, P., & Rutherford, A. W. (1989) *Biochim. Biophys. Acta 977*, 207–214.
Van Mieghem, F. J. E., Satoh, K., & Rutherford, A. W. (1991) *Biochim. Biophys. Acta 1058*, 379–385.
Vass, I., & Styring, S. (1991) *Biochemistry 30*, 830–839.
Velthuys, B. (1981) *FEBS Lett. 126*, 277–281.
Vermaas, W. F. J., & Rutherford, A. W. (1984) *FEBS Lett. 175*, 243–248.
Vermaas, W. F. J., Rutherford, A. W., & Hansson, Ö. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8477–8481.
Vermaas, W. F. J., Charité, J., & Shen, G. (1990a) *Biochemistry 29*, 5325–5332.
Vermaas, W. F. J., Charité, J., & Shen, G. (1990b) *Z. Naturforsch. 45*, 359–365.
Wraight, C. A. (1981) *Isr. J. Chem. 21*, 348–354.
Yocum, C. F. (1991) *Biochim. Biophys. Acta 1059*, 1–15.
Zimmermann, J.-L., & Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 169–167.
Zimmermann, J.-L., & Rutherford, A. W. (1986) *Biochemistry 25*, 4609–4615.

Synthesis and Biochemical Characterization of the New Sulfhydryl-Reactive ATP Analogue 8-Thiocyano-ATP. Its Interaction with Na,K-ATPase and Kinases†

Georgios Scheiner-Bobis,*,‡ Wolfgang Mertens, Martin Willeke, and Wilhelm Schoner
Institut für Biochemie, Fachbereich Veterinärmedizin und Tierzucht, Justus-Liebig-Universität Giessen, Frankfurter Strasse 100, D-6300 Giessen, Germany

Received October 1, 1991; Revised Manuscript Received November 20, 1991

ABSTRACT: The synthesis of 8-thiocyano-ATP (CNS^8-ATP) is described. At 37 °C the ATP analogue inactivates Na,K-ATPase, hexokinase, and pyruvate kinase. In all three cases, inactivation can be prevented by the addition of ATP, thus indicating that CNS^8-ATP is recognized within the ATP binding site of the above enzymes. Incubation of the inactivated enzymes with dithiothreitol restores the catalytic activities. Therefore, it is likely that in these enzymes a mixed disulfide (E-S-S^8-ATP) is formed between a sulfhydryl in the ATP binding site (E-SH) and the ATP analogue:

$$\text{E-SH} + \text{CNS}^8\text{-ATP} \underset{K_D}{\leftrightarrow} [\text{E-SH}\cdot\text{CNS}^8\text{-ATP}] \xrightarrow[k_2]{} \text{E-S-S}^8\text{-ATP} + \text{HCN}$$

From the pseudo-first-order inactivation kinetics, a $K_D = 2.7\ \mu M$ with $k_2 = 0.142\ min^{-1}$ is calculated for the hexokinase and a $K_D = 40\ \mu M$ with $k_2 = 0.347\ min^{-1}$ is calculated for the pyruvate kinase interactions with the ATP analogue. At 4 °C, Na,K-ATPase recognizes CNS^8-ATP with a $K_D = 8.3\ \mu M$. At 37 °C, the enzyme becomes inactivated by the ATP analogue in a biphasic manner. Inactivation results in the incorporation of [α-^{32}P]8-CNS^8-ATP into the catalytic α-subunit of the enzyme. Limited tryptic digestion in the presence of 150 mM KCl results in the formation of a radioactive peptide of $M_r = 56\,000$, known to bear the purine binding domain of Na,K-ATPase. The results described in this article verify CNS^8-ATP as a sulfhydryl-reactive ATP analogue and characterize this new ATP analogue as a useful tool for structure/function studies on ATP-recognizing enzymes.

The catalytic α-subunit of Na,K-ATPase[1] contains 23 cyst(e)ine residues (Shull et al., 1985). At least one of them is thought to be localized within or very close to the ATP binding site of the enzyme (Skou, 1974; Patzelt-Wenczler et al., 1975; Jesaitis & Fortes, 1980; Schoner et al., 1987). This conclusion is based upon experiments performed with *N*-ethylmaleimide or sulfhydryl-reactive analogues of 6-

† This work was supported by the Deutsche Forschungsgemeischaft through SFB 249, Pharmakologie Biologischer Makromoleküle and the Fonds der Chemischen Industrie, Frankfurt/M.

* Address correspondence to this author.
‡ Current address: Department of Physiology and Biophysics, University of Southern California School of Medicine, 1333 San Pablo Street, Los Angeles, CA 90033.

[1] Abbreviations and enzymes: CNS^8-ATP, 8-thiocyanoadenosine 5′-triphosphate (8-thiocyano-ATP); 8-BrATP, 8-bromoadenosine 5′-triphosphate; 8-SH-ATP, 8-mercaptoadenosine 5′-triphosphate; 8-SH-ADP, 8-mercaptoadenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; SDS, sodium dodecyl sulfate; Na,K-ATPase, sodium and potassium activated adenosine 5′-triphosphatase (EC 3.6.1.37); hexokinase, ATP:D-hexose 6-phosphotransferase (EC 2.7.1.1); pyruvate kinase, ATP:pyruvate 2-*O*-phosphotransferase (EC 2.7.1.40).

0006-2960/92/0431-2107$03.00/0

mercaptoinosine where it was demonstrated that in both cases inhibition of Na,K-ATPase can be prevented by ATP (Schoner et al., 1987). The peptide, however, carrying the sulfhydryl group essential for ATP recognition has not be identified. The difficulty in applying maleimide derivatives could be that their use cannot exclude the possibility of nonspecific labeling of cysteine groups outside the catalytic site (Le, 1986). The derivatives of 6-mercaptoinosine, on the other hand, have a relatively low affinity for the Na,K-ATPase, probably because they lack the 6-NH_2 group of the purine (Schoner et al., 1968; Schuurmans Stekhoven et al., 1986). Additionally, the synthesis of the 6-mercaptoinosine derivatives in a ^3H-, ^{14}C-, or α-^{32}P-labeled 5'-triphosphate form, which would be absolutely necessary for labeling experiments, is very laborious (Schoner et al., 1987) and would require the handling of relatively large amounts of radioactivity in order to compensate the quite low affinity of these compounds toward Na,K-ATPase.

In order to circumvent these difficulties regarding specificity or preparative procedures, we synthesized a new sulfhydryl-reactive ATP analogue, 8-thiocyano-ATP (CNS^8-ATP), which retains the 6-NH_2 group of its purine. In the work presented here, we investigate the mode of action and specificity of the reaction of CNS^8-ATP toward Na,K-ATPase, hexokinase, and pyruvate kinase, enzymes that use ATP as a substrate. As we demonstrate in this paper, CNS^8-ATP forms mixed disulfides with cysteine groups localized within the ATP binding sites of Na,K-ATPase, hexokinase, or pyruvate kinase and inactivates all three enzymes. The mixed disulfide formed with the Na,K-ATPase is localized on the M_r = 56 000 tryptic peptide known to contain the purine binding subsite of the ATP binding site of the enzyme (Ponzio et al., 1983; Scheiner-Bobis & Schoner, 1985; Farley et al., 1985). In similar experiments, CNS^8-ATP could therefore be used to label and localize sulfhydryl groups within the ATP binding site of other ATP-recognizing enzymes. The data obtained here describe and characterize the new probe as a useful tool in structure/function studies for ATPases and kinases.

Materials and Methods

Materials. Yeast hexokinase and rabbit muscle pyruvate kinase were purchased from Boehringer (Mannheim). [α-^{32}P]ATP (400 Ci/mmol) was from Amersham Buchler (Braunschweig). Chemicals for electrophoresis and molecular weight markers were obtained from Serva (Heidelberg). PEI cellulose was from Schleicher and Schuell (Dassel). All other chemicals were of analytical grade.

Purification of Na,K-ATPase. Na,K-ATPase was isolated from sheep kidneys by the method described earlier (Jørgensen, 1974). The specific activity of the enzyme, verified by a coupled spectrophotometric assay (Scheiner-Bobis & Schoner, 1985) and by the protein determination method of Lowry et al. (1951) with the modifications described (Scheiner-Bobis & Schoner, 1985), was in the range of 25–30 units/mg of protein.

Synthesis of CNS^8-ATP. 8-Thiocyano-ATP was synthesized in a three-step procedure. The starting material is 8-BrATP, the synthesis of which from ATP has already been described (Scheiner-Bobis & Schoner, 1985; Ikehara & Uesugi, 1969).

In order to obtain the tri-*N*-butylammonium form of 8-BrATP, 650 μmol of 8-BrATP (tri-*N*-ethylammonium form) was suspended in 30 mL of pyridine containing 0.2 mL of tri-*N*-butylamine. The mixture was evaporated to dryness in a rotary evaporator. The ATP analogue was resuspended in 50 mL of pyridine twice with subsequent rotary evaporation. Remaining pyridine was removed by 2-fold resuspension and rotary evaporation of 8-BrATP in 50 mL of dry DMF.

The 8-BrATP (tri-*N*-butylammonium salt) was converted to 8-SH-ATP as described earlier (Feist & Cramer, 1978). This method yields 8-SH-ADP as the main product. The 8-SH-ADP was converted to 8-SH-ATP with an 80% yield by incubation of 1 mM 8-SH-ADP at 37 °C for 2 h with 20 mM potassium phosphate, pH 7.5/8 mM $MgCl_2$/80 mM KCl/2 mM phosphoenolpyruvate/20 units of rabbit muscle pyruvate kinase. The substances were easily separated from each other by DEAE-Sephadex A-25 (HCO_3^-) chromatography using triethylamine (pH 7.2) as elution buffer in a linear gradient from 0.0 to 0.8 M.

Synthesis of CNS^8-ATP from 8-SH-ATP was carried out on ice in a fume hood. A total of 50 μmol of 8-SH-ATP (tri-*N*-ethylammonium form) was dissolved in 0.58 mL of water, and 51 μL of 1 N NaOH was added. A total of 5.46 mg of BrCN dissolved in 149 μL of ethanol was added under continuous stirring to the above mixture in portions of 14.9 μL within 20 min. The mixture was allowed to stay on ice for an additional 20 min. The conversion of 8-SH-ATP to CNS^8-ATP was then complete. CNS^8-ATP was separated from smaller reactants by Sephadex G-10 chromatography, using water as eluent.

The synthesis of [α-^{32}P]CNS^8-ATP was performed after dilution of 1 mCi of [α-^{32}P]ATP with 0.1 mmol of ATP following the above procedure. The synthesis of [α-^{32}P]8-BrATP has been described (Scheiner-Bobis & Schoner, 1985).

The product of the above synthesis, CNS^8-ATP, was analyzed by ^{13}C- and ^1H-NMR spectroscopy. As expected, these analytic methods showed the replacement of the proton from the 8-SH group by the cyano group. CNS^8-ATP has an absorbance maximum at 278 nm, which is different from the absorbance maximum of 8-SH-ATP at 298 nm. It migrates as a single spot on PEI cellulose with a R_f value of 0.36 when 0.3 M KH_2PO_4 is used as solvent. The molecular absorbance for CNS^8-ATP was determined to be ϵ = 26 640 L·mol^{-1}·cm^{-1} (Mejbaum, 1939).

Interaction of CNS^8-ATP with amino acids was monitored at room temperature by measuring the ultraviolet spectra of aqueous solutions of 40 μM CNS^8-ATP and 150 μM either alanine, serine, glutamic acid, lysine, or cysteine. The reaction of CNS^8-ATP with cysteine results in the liberation of cyanide from the ATP analogue, which can qualitatively be identified as Berlin blue (Blitz et al., 1976).

Interactions of CNS^8-ATP with Na,K-ATPase. The affinity of CNS^8-ATP for Na,K-ATPase was determined at 4 °C by displacing [α-^{32}P]ATP from the catalytic site of the enzyme (Jensen & Nørby, 1971). Additionally, the apparent dissociation constant of the ATP analogue·Na,K-ATPase complex was determined from ability of CNS^8-ATP to displace eosin Y from the high-affinity ATP binding site of the enzyme. These studies were carried out at 4 °C, using 45 μg of Na,K-ATPase in 40 mM histidine hydrochloride, pH 7.25/100 mM Na_2EDTA/20 mM NaCl/1 μM eosin Y (Skou & Esmann, 1988). Changes in fluorescence were monitored in a Hitachi F-3000 spectrofluorometer at an emission of 560 nm, using an excitation at 530 nm. The bandpasses were each 5 nm.

Inactivation of Na,K-ATPase by the ATP analogue performed at 37 °C by incubating 1.85 units of the enzyme in 10 mM Tris-HCl, pH 7.25/5 mM $MgCl_2$ and increasing concentrations of CNS^8-ATP. The total volume of the samples was 0.5 mL. The time course of the remaining activity of Na,K-ATPase was followed in 30-μL aliquots of the samples by a coupled spectrophotometric assay (Scheiner-Bobis &

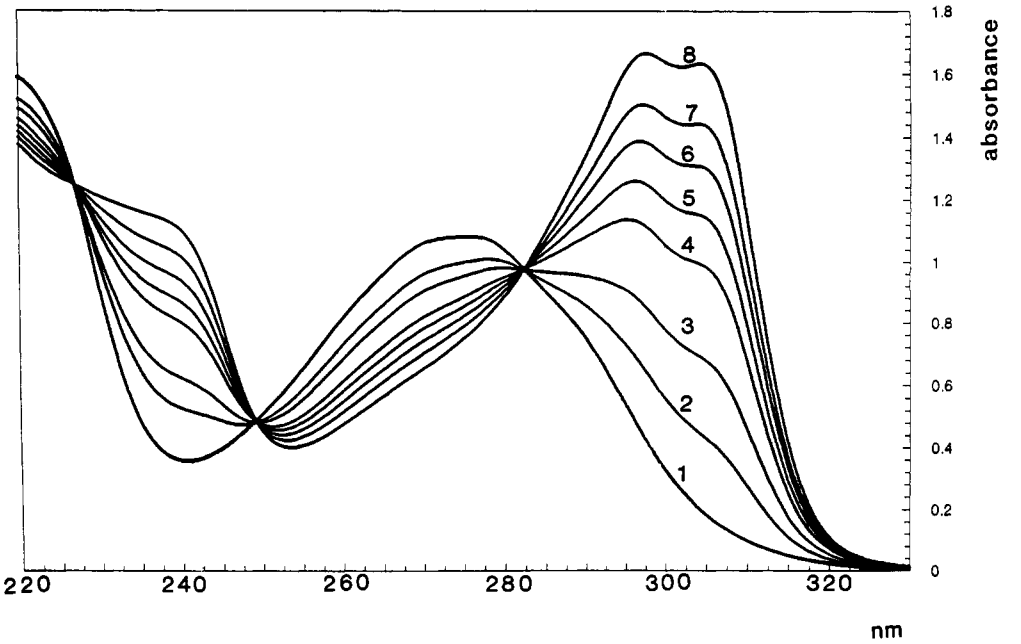

FIGURE 1: Change of the spectral properties of CNS^8-ATP upon incubation with cysteine. A 150 μM concentration of cysteine was incubated at room temperature with 40 μM CNS^8-ATP. The changes of the spectrum were followed for 30 min (1 = 0 min, 2 = 1 min, 3 = 3 min, 4 = 5 min, 5 = 10 min, 6 = 15 min, 7 = 20 min, and 8 = 30 min). The shift of the maximum absorbance from 277 to 305 nm is accompanied by the release of cyanide which can be determined qualitatively as Berlin blue (Blitz et al., 1976).

Schoner, 1985). The conditions for the effects of ATP on the inactivation are indicated in the legend of Figure 4.

Reactivation of 6 units of Na,K-ATPase inactivated for 90 min at 37 °C by 100 μM CNS^8-ATP was achieved by the addition of 20 mM dithiothreitol to the mixture. The regeneration of the activity of the enzyme treated with dithiothreitol and the activity of the control without dithiothreitol were monitored over a period of 250 min by the coupled spectrophotometric assay (Scheiner-Bobis & Schoner, 1985). Labeling of 500 μg of Na,K-ATPase with 100 μM [α-^{32}P]-CNS^8-ATP (6.8 μCi/nmol at the day of use) was carried out at 37 °C in 10 mM Tris-HCl, pH 7.25/5 mM $MgCl_2$/and 10 mM NaCl. The final volume was 5 mL. Thereafter, the enzyme was centrifuged at 100000g for 30 min and the pellet was resuspended in 1 mL of 25 mM imidazole hydrochloride, pH 7.25. After trypsinolysis under controlled conditions in the presence of 150 mM KCl (Giotta, 1975; Castro & Farley, 1979), 100-μg aliquots of the labeled enzyme were run on SDS–polyacrylamide slab gels containing 7.5% acrylamide and 0.3% *N,N'*-methylenebisacrylamide, prepared according to Laemmli (1970). β-Mercaptoethanol was omitted. The dried gel was exposed for 9 days at −60 °C to a Kodak X-Omat X-ray film.

Inactivation of Hexokinase by CNS^8-ATP. A total of 6.7 units of hexokinase was incubated at 37 °C with increasing CNS^8-ATP in 250 μL of 20 mM triethanolamine hydrochloride pH 7.6. The activity of the enzyme was determined in 20-μL aliquots by a coupled spectrophotometric assay (Bergmeyer, 1974). The protective effect of ATP against inactivation was studied by incubating 6.7 units of hexokinase with 167 μM CNS^8-ATP and increasing concentrations of ATP. All other conditions were the same as described above.

Reactivation of 6.7 units of hexokinase inactivated to 5% activity by 167 μM CNS^8-ATP was followed over a period of 40 min after the addition of 7.7 mM dithiothreitol.

Inactivation of Pyruvate Kinase by CNS^8-ATP. A total of 6.3 units of pyruvate kinase was incubated in 250 μL of 40 mM Tris-HCl, pH 7.25, with increasing CNS^8-ATP. The protective effect of ATP against the inactivation was studied at 40 μM CNS^8-ATP. The activity course of the enzyme was monitored by a coupled spectrophotometric assay (Noda et al., 1986).

Results

Mode of Action of CNS^8-ATP in the Inactivation of Na,K-ATPase. CNS^8-ATP has a maximum absorbance at 277 nm (Figure 1). Incubation of the analogue with cysteine leads to the formation of a new absorbance maximum at 305 nm with a shoulder at 310 nm and three isosbestic points at 227, 249, and 282 nm (Figure 1). These changes of the absorbance spectrum, which do not occur upon the exposition of CNS^8-ATP to amino acids other than cysteine, are accompanied by the release of CN^-, which can be detected as Berlin blue in a qualitative assay for cyanide after its transformation to sodium cyanoferrate (Blitz, 1976). Therefore, it is likely that CNS^8-ATP reacts with sulfhydryl groups by simultaneous release of CN^- anions. When increasing concentrations of CNS^8-ATP are incubated at 4 °C with Na,K-ATPase and [α-^{32}P]ATP in the presence of EDTA (Jensen & Nørby, 1971), the analogue displaces the radioactive ATP from the ATP binding site of the enzyme (not shown). From these ATP displacement studies, an apparent dissociation constant of K_D = 8.3 μM could be determined for the complex formed between CNS^8-ATP and Na,K-ATPase. Competition studies between the ATP analogue and eosin Y, which binds to the high-affinity ATP binding site of Na,K-ATPase, reveal an apparent dissociation constant of 5.9 μM for CNS^8-ATP (Figure 2). Both these values show that the ATP analogue competes with ATP at the high-affinity ATP binding site of the enzyme (Hegyvary & Post, 1971; Skou & Esmann, 1988).

At 37 °C and in the presence of Mg^{2+}, CNS^8-ATP inactivates Na,K-ATPase (Figure 3). The inactivation is biphasic, a pattern also observed in earlier studies on the inactivation of Na,K-ATPase with sulfhydryl reactive analogues of inosine 5'-triphosphate (Patzelt-Wenzler et al., 1975; Patzelt-Wenzler & Mertens, 1981; Koepsell et al., 1982; Schoner et al., 1987). The activity of Na,K-ATPase inactivated by CNS^8-ATP can be restored by treatment of the enzyme with dithiothreitol

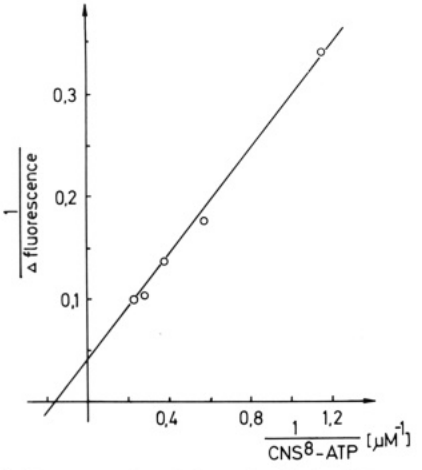

FIGURE 2: Displacement of eosin from the high-affinity ATP binding site of Na,K-ATPase by CNS^8-ATP. Increasing concentrations of CNS^8-ATP were used to displace eosin from the high-affinity ATP binding site of Na,K-ATPase (see Materials and Methods). At 4 °C the double-reciprocal plot of the obtained fluorescence versus the concentration of the analogue reveals an apparent K_D of 5.9 μM.

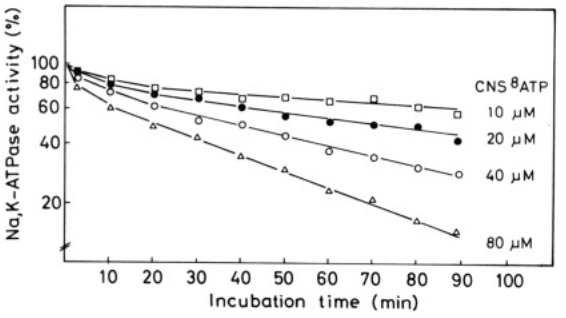

FIGURE 3: Inactivation of Na,K-ATPase by CNS^8-ATP. A total of 1.85 units of Na,K-ATPase was incubated at 37 °C in 10 mM Tris-HCl, pH 7.25/5 mM $MgCl_2$ with increasing concentrations of CNS^8-ATP. The remaining activity of the enzyme was followed over time by transferring aliquots of 30 μL from the reaction mixture to a coupled spectrophotometric assay.

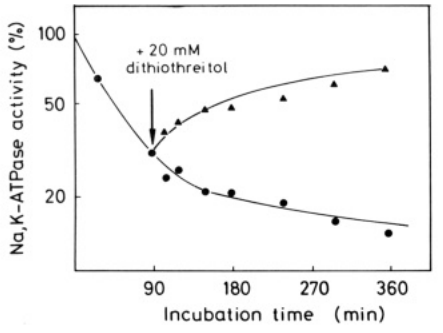

FIGURE 4: Effect of dithiothreitol on the CNS^8-ATP inactivated Na,K-ATPase. A total of 6 units of Na,K-ATPase was inactivated for 90 min by 100 μM CNS^8-ATP. Thereafter, 20 mM dithiothreitol was added to half of the reaction mixture, and the other half served as a control.

(Figure 4). Since dithiothreitol reduces and splits disulfide bridges to sulfhydryl groups, it is likely that inactivation of Na,K-ATPase by CNS^8-ATP is due to the formation of a mixed disulfide (E-S-S^8-ATP) between a cysteine sulfhydryl group of the enzyme (E-SH) and the analogue (eq 1). This

$$\text{E-SH} + \text{CNS}^8\text{-ATP} \leftrightarrow \text{E-S-S}^8\text{-ATP} + \text{HCN} \quad (1)$$

conclusion is also supported by studies on the interaction of the ATP analogue with amino acids (Figure 1) and by the fact that CN^- can be detected after incubation of CNS^8-ATP with cysteine.

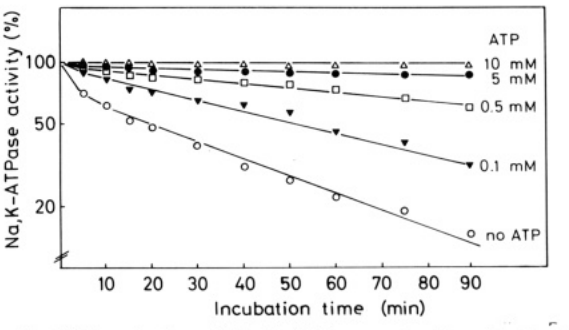

FIGURE 5: ATP protection of Na,K-ATPase against inactivation by CNS^8-ATP. A total of 1.85 units of Na,K-ATPase was incubated in 10 mM Tris-HCl, pH 7.25 with 40 μM CNS^8-ATP, 20 mM $MgCl_2$, and increasing concentrations of ATP.

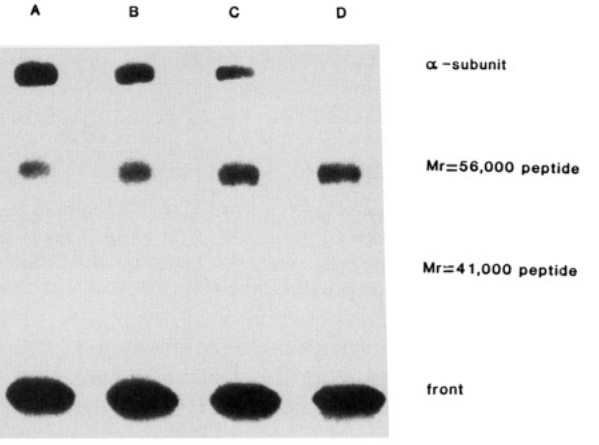

FIGURE 6: Autoradiography of Na,K-ATPase labeled with [α-^{32}P]-CNS^8-ATP. A total of 500 μg of Na,K-ATPase was labeled by 100 μM [α-^{32}P]CNS^8-ATP in the presence of 5 mM $MgCl_2$ and 10 mM NaCl. After trypsinolysis for 5, 10, 15, and 20 min (lanes A–D) under controlled conditions in the presence of 150 mM KCl, aliquots of 100 μg of labeled peptides were separated on a SDS–polyacrylamide slab gel (Laemmli, 1970). β-Mercaptoethanol was omitted. The dried gel was exposed at −60 °C for 9 days to a Kodak X-Omat film. It is obvious from the results shown in the figure that all the label is associated with the M_r = 56 000 peptide, which is formed upon tryptic cleavage of the catalytic α-subunit in the presence of K^+. This peptide is known to recognize the adenosine part of ATP. The simultaneously formed M_r = 41 000 peptide (its position is indicated in the figure) does not contain any of the label. These results make it very likely that CNS^8-ATP modifies the purine-recognizing moiety of the catalytic α-subunit of the sodium pump.

Figure 5 demonstrates that ATP completely protects Na,K-ATPase against the inactivation by CNS^8-ATP, supporting the hypothesis that the modification of sulfhydryl groups occurs within the ATP binding site of Na,K-ATPase. Additional support for this hypothesis is the labeling of the catalytic α-subunit of Na,K-ATPase by [α-^{32}P]CNS^8-ATP (Figure 6). This figure also shows the formation of a radioactive tryptic peptide with M_r = 56 000 derived from the catalytic α-subunit. This fragment of the α-subunit has been suggested to participate in the adenosine binding domain of the ATP binding site of Na,K-ATPase (Farley et al., 1985; Scheiner-Bobis & Schoner, 1985; Ohta et al., 1986). The simultaneously formed M_r = 41 000 peptide (its position is indicated in the same figure) remains unlabeled.

Inactivation of Hexokinase and Pyruvate Kinase by CNS^8-ATP. In contrast to the biphasic inactivation pattern observed in the studies with the Na,K-ATPase, inactivation of hexokinase or pyruvate kinase by CNS^8-ATP follows first-order reaction characteristics (not shown). By plotting the apparent inactivation half-life τ for the inactivation of hexokinase or pyruvate kinase at different concentrations of

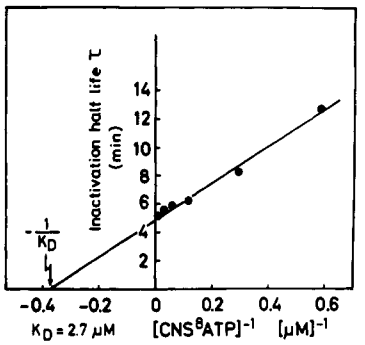

FIGURE 7: Inactivation of yeast hexokinase by CNS^8-ATP. A total of 6.7 units of hexokinase in 250 μL of 20 mM triethanolamine, pH 7.6, was incubated at 37 °C with increasing concentrations of CNS^8-ATP. This plot is of the apparent inactivation half-life τ as a function of the reciprocal concentration of the ATP analogue.

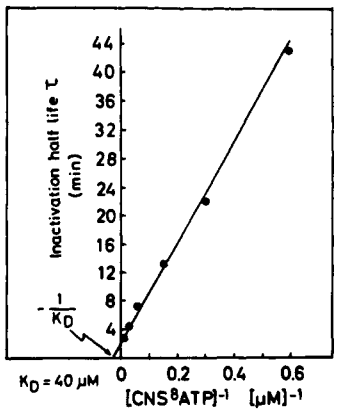

FIGURE 8: Inactivation of rabbit muscle pyruvate kinase. A total of 6.3 units of pyruvate kinase was incubated at 37 °C in 250 μL of 40 mM Tris-HCl, pH 7.25, with increasing concentrations of CNS^8-ATP. The remaining activity of the enzyme was determined in a coupled spectrophotometric assay. This plot is of the apparent inactivation half-life τ against the reciprocal concentration of CNS^8-ATP.

CNS^8-ATP against the reciprocal concentration of the ATP analogue, in both cases straight lines are obtained (Figures 7 and 8). Extrapolation of these lines to infinite concentrations of CNS^8-ATP yields an inactivation half-life of τ = 4.85 min for the hexokinase inactivation and of τ = 2.00 min for the pyruvate kinase inactivation (Figures 7 and 8). Additionally, the data obtained in Figures 7 and 8 indicate that in both cases the process of inactivation is preceded by the formation of a dissociable enzyme·CNS^8-ATP complex (Mares-Guia & Shaw, 1967; Chen & Engel, 1975). Since dithiothreitol restores the activity of hexokinase to 95% (Figure 9) and the activity of pyruvate kinase to 80% (not shown), the inactivation of both enzymes can be described by eq 2. The dissociation

$$\text{E-SH} + \text{CNS}^8\text{-ATP} \underset{K_D}{\leftrightarrow} [\text{E-SH·CNS}^8\text{-ATP}] \xrightarrow[k_2]{} \text{E-S-S}^8\text{-ATP} + \text{HCN} \quad (2)$$

constants between the ATP analogue and hexokinase or pyruvate kinase were calculated from the intercept of the straight lines with the abscissa (Figures 7 and 8) as K_D = 2.7 μM and K_D = 40 μM, respectively (Lasch, 1987). The inactivation rate constants k_2 were calculated from the intercept with the ordinate ($\tau = (\ln 2)k_2^{-1}$) as k_2 = 0.143 min^{-1} for the hexokinase reaction (Figure 7) and k_2 = 0.347 min^{-1} for the pyruvate kinase reaction (Figure 8) (Lasch, 1987). The inactivation of both hexokinase and pyruvate kinase can be hindered by ATP (not shown). At 167 μM CNS^8-ATP and in the absence of ATP, the first-order inactivation rate k_i for the

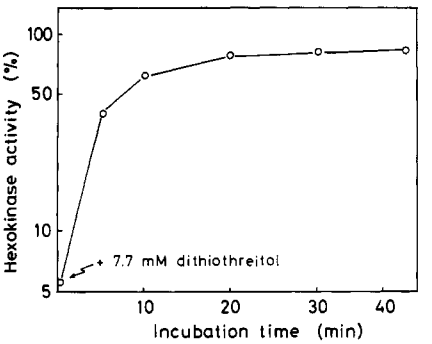

FIGURE 9: Reactivation of CNS^8-ATP-inactivated hexokinase by dithiothreitol. A total of 6.7 units of hexokinase was inactivated to 95% by 167 μM CNS^8-ATP. Thereafter, 7.7 mM dithiothreitol was added to the reaction mixture.

inactivation of hexokinase is 0.116 min^{-1}. Addition of 3.3 mM ATP causes a decrease of the rate to k_i = 0.03 min^{-1}, resulting in the protection of the enzyme against inactivation. At 40 μM CNS^8-ATP, the k_i for the inactivation of the pyruvate kinase changes from 0.141 min^{-1} to 0.05 min^{-1} when 3.3 mM ATP is present in the inactivation mixture. These findings demonstrate that CNS^8-ATP is an affinity label for sulfhydryl groups localized within the purine recognition site of both enzymes.

Discussion

The aim of the work described here was the synthesis of a covalent, sulfhydryl-reactive ATP analogue which can be used for labeling experiments and kinetic analysis of the reaction mechanism of ATPases and kinases. It was further desired to start the synthesis from ATP, which can be purchased in various radioactive forms. All the known sulfhydryl-reactive ATP analogues used so far are derivatives of 6-mercaptoinosine, the synthesis of which as triphosphates is time consuming and requires the handling of considerable amounts of radioactive phosphate to prepare them in a radioactive form (Schoner et al., 1987). The synthesis of ^{3}H- or ^{14}C-labeled sulfhydryl-reactive derivatives of 6-mercaptoinosine is even more expensive and laborious. CNS^8-ATP can be synthesized starting from ATP. Its exposure to cysteine leads to the change of its spectral properties (Figure 1) and results in the liberation of CN$^-$, probably via the formation of a cysteinyl-S^8-ATP disulfide. At 37 °C, CNS^8-ATP inactivates Na,K-ATPase (Figure 3), hexokinase (Figure 7), and pyruvate kinase (Figure 8). Considering that the activity of the inactivated enzymes can be restored by the addition of dithiothreitol (Figures 4 and 9), a substance known to split disulfide bonds, it seems likely that the inactivation is due to the formation of a mixed disulfide between the CNS^8-ATP and a cysteine residue of the enzymes. The formation of a complex between the α-subunit of Na,K-ATPase and [α-^{32}P]CNS^8-ATP, which in the absence of β-mercaptoethanol is stable enough to survive electrophoresis (Figure 6), supports the idea of a mixed disulfide formed between enzyme and ATP analogue during inactivation.

The inactivation kinetics of hexokinase and pyruvate kinase can be described by pseudo-first-order reactions. In both cases, the time and concentration dependence of inactivation results in straight lines in a semilogarithmic plot (not shown). A graph of the apparent inactivation half-life τ obtained at different concentrations of the ATP analogue versus the reciprocal concentration of the ATP analogue is a straight line, which indicates that at an infinite concentration of the inhibitor the inactivation half-life τ reaches a maximal value of τ = 4.85 min for hexokinase and τ = 2.00 min for the pyruvate kinase.

This indicates that the formation of the mixed disulfide between CNS^8-ATP and the enzymes is preceded by the formation of a dissociable [enzyme·CNS^8-ATP] complex (Mares-Guia & Shaw, 1967) (eq 2). The dissociation constants are K_D = 2.7 μM for the hexokinase·CNS^8-ATP complex (Figure 7) and K_D = 40 μM for the pyruvate kinase·CNS^8-ATP complex (Figure 8). The rate constant k_2, which can be estimated from the intercept with the ordinate of the straight lines in Figures 7 and 8 as $k_2 = (\ln 2)\tau^{-1}$, indicates for both enzymes a rather slow inactivation step (k_2 = 0.143 min^{-1} for the hexokinase and k_2 = 0.347 min^{-1} for the pyruvate kinase reaction).

In contrast to the single-exponential inactivation curves obtained with hexokinase and pyruvate kinase, the inactivation of Na,K-ATPase follows a more complicated mode, expressed in biphasic curves (Figures 3 and 5). The results obtained with hexokinase and pyruvate kinase indicate that these biphasic kinetics are not likely to be a property of the ATP analogue, but rather a characteristic property of Na,K-ATPase. Comparable kinetics obtained in inactivation studies of Na,K-ATPase with sulfhydryl-reactive derivatives of 6-mercaptoinosine 5′-triphosphate have been preferentially interpreted to indicate the reaction of the inhibitors with two different sulfhydryl groups (Fritsch & Koepsell, 1983; Fritsch, 1985). The rapid phase of the reaction has been interpreted to indicate the reaction of the sulfhydryl-reactive probe with a cysteine group in the high-affinity ATP binding site of the enzyme; the slow part is ascribed to the interaction of the analogue with a cysteine from the low-affinity ATP binding site (Patzelt-Wenczler & Schoner, 1981; Fritsch & Koepsell, 1983; Fritsch, 1985). This might also be true for CNS^8-ATP, and it will be interesting to see whether the mathematical inactivation model developed by Fritsch and Koepsel (1983) and Fritsch (1985) will fit the data obtained with CNS^8-ATP. In all three cases, however, modification of sulfhydryl groups probably occurs within the ATP binding site of Na,K-ATPase (Figure 5), hexokinase, and pyruvate kinase, since ATP protects all three enzymes against inactivation by CNS^8-ATP. The demonstration of a sulfhydryl group within the ATP binding site of pyruvate kinase by CNS^8-ATP is not unexpected. Bezares et al. (1968) showed in their experiments with the 2′,3′-dialdehyde of ADP that a cysteine is a part of the peptide labeled by this ADP analogue. It is possible that CNS^8-ATP is attached to the same peptide.

The conclusion that CNS^8-ATP is an ATP binding site directed probe is supported also by the ability of the analogue at 4 °C to replace [α-^{32}P]ATP from the high-affinity ATP binding site of Na,K-ATPase with a K_D = 8.3 μM or eosin with an apparent K_D of 5.9 μM (Figure 2). These values are 2–10 times lower than the K_D values of the 6-mercaptoinosine 5′-triphosphate derivatives ranging between 20 and 70 μM. The dissociation constant for ATP at the high-affinity ATP binding site is 0.1–2 μM (Schoner et al., 1987). The K_D value for CNS^8-ATP is in the range of enzyme–substrate complexes of 8-bromo-ATP and 8-azido-ATP (Schoner et al., 1987). The higher affinity of the Na,K-ATPase for CNS^8-ATP as compared with the 6-mercapto-ITP derivatives can probably be ascribed to the existence of the 6-amino group of the purine of CNS^8-ATP. ITP and GTP, which lack this group, can phosphorylate the enzyme, but they are rather weak substrates of Na,K-ATPase (Schoner et al., 1968; Schuurmans Stekhoven et al., 1986).

A final proof for the ATP binding site directed interaction of the CNS^8-ATP is derived from the labeling and digestion experiments of Na,K-ATPase (Figure 6). After digestion of the enzyme with trypsin, a M_r = 56 000 labeled peptide, derived from the α-subunit, is formed. This fragment, which occurs upon trypsinolysis in the presence of 150 mM KCl, contains the adenosine-recognizing moiety of the Na,K-ATPase, since it is labeled with all ATP analogues described so far (Farley et al., 1985; Scheiner-Bobis & Schoner, 1985; Ohta et al., 1986). The data obtained in these studies allow the conclusion that CNS^8-ATP is an ATP analogue interacting with and covalently modifying cysteine residues within the catalytic site of Na,K-ATPase, hexokinase, and pyruvate kinase. It probably will be a useful tool for the labeling and identification of cysteine-containing peptides derived from the ATP binding site of these enzymes. Work regarding the isolation of a tryptic peptide labeled in the catalytic site of Na,K-ATPase is in progress. The use of this ATP analogue with other ATP-recognizing enzymes will help to understand structure/function relationships and will show if ATP recognition follows generally manifested principles.

Acknowledgments

G. Scheiner-Bobis would like to thank Dr. Robert A. Farley and Dr. Edward E. Huston for helpful discussions.

References

Bergmeyer, H. U. (1974) *Methoden der Enzymatischen Analyse*, pp 502–503, Verlag Chemie, Weinheim/Bergstr.

Bezares, G., Euzaguirre, J., Hinrichs, M. V., Heinrikson, R. L., Reardon, I., Kemp, R. G., Latsha, S. P., & Bazaes, S. (1987) *Arch. Biochem. Biophys. 253*, 133–137.

Blitz, Klemm, & Fischer (1976) Experimentele Einführung in die Anorganische Chemie, pp 171–172, De Gruyter, Berlin, New York.

Castro, J., & Farley, R. A. (1979) *J. Biol. Chem. 254*, 2221–2228.

Chen, S., & Engel, P. C. (1975) *Biochem. J. 147*, 351–358.

Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1985) *J. Biol. Chem. 259*, 9532–9535.

Feist, W., & Cramer, F. (1978) *Nucleic Acid Synthesis* (Townsend, L. B., & Tipson, S. R., Eds.) pp 827–836, John Wiley & Sons, Inc., Chichester.

Fritsch, G. K. (1985) *J. Theor. Biol. 117*, 397–415.

Fritsch, G. K., & Koepsell, H. (1983) *J. Theor. Biol. 102*, 469–476.

Giotta, G. J. (1975) *J. Biol. Chem. 250*, 5159–5164.

Hegyvary, C., & Post, R. L. (1971) *J. Biol. Chem. 246*, 5234–5240.

Ikehara, M., & Uesugi, S. (1969) *Chem. Pharm. Bull. 17*, 348–354.

Jensen, J., & Nørby, J. G. (1971) *Biochim. Biophys. Acta 233*, 395–403.

Jesaitis, A. J., & Fortes, P. A. G. (1980) *J. Biol. Chem. 255*, 459–467.

Jørgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.

Koepsell, H., Hulla, F. W., & Fritsch, G. (1982) *J. Biol. Chem. 257*, 10733–10741.

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

Lasch, J. (1987) *Enzymkinetik*, pp 64–67, Springer-Verlag, Berlin, New York, Tokyo.

Le, T. D. (1986) *Biochemistry 25*, 2379–2386.

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

Mares-Guia, M., & Shaw, E. (1967) *J. Biol. Chem. 242*, 5782–5788.

Mejbaum, W. (1939) *Z. Physiol. Chem. 145*, 93–106.

Noda, S., Horn, F., Linder, D., & Schoner, W. (1986) *Eur. J. Biochem. 155*, 643–651.

Ohta, T., Nagano, K., & Yoshida, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2071–2075.
Patzelt-Wenczler, R., & Mertens, W. (1981) *Eur. J. Biochem. 121*, 197–202.
Patzelt-Wenczler, R., & Schoner, W. (1981) *Eur. J. Biochem. 114*, 79–87.
Patzelt-Wenczler, R., Pauls, H., Erdmann, E., & Schoner, W. (1975) *Eur. J. Biochem. 53*, 301–311.
Ponzio, G., Rossi, B., & Lazdunski, M. (1983) *J. Biol. Chem. 258*, 8201–8205.
Scheiner-Bobis, G., & Schoner, W. (1985) *Eur. J. Biochem. 152*, 739–746.
Schoner, W., Beusch, R., & Kramer, R. (1968) *Eur. J. Biochem. 7*, 102–110.
Schoner, W., Scheiner-Bobis, G., Hasselberg, M., Rempeters, G., & Pauls, H. (1987) *Top. Mol. Pharmacol. 4*, 97–127.
Schuurmans Stekhoven, F. M. A. H., Swarts, H. G. P., Zhao, R. S., & de Pont, J. J. H. H. M. (1986) *Biochim. Biophys. Acta 861*, 259–266.
Shull, G. E., Schwarz, A., & Lingrell, J. B. (1985) *Nature 316*, 691–695.
Skou, J. C. (1974) *Biochim. Biophys. Acta 339*, 234–245.
Skou, J. C., & Esmann, M. (1988) *Methods Enzymol. 156*, 278–287.

5-Chlorolevulinate Modification of Porphobilinogen Synthase Identifies a Potential Role for the Catalytic Zinc†

Eileen K. Jaffe,* William R. Abrams, Henry X. Kaempfen, and Kenneth A. Harris, Jr.

Biochemistry Department, University of Pennsylvania School of Dental Medicine, 4001 Spruce Street, Philadelphia, Pennsylvania 19104-6002, and Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111

Received September 12, 1991; Revised Manuscript Received November 15, 1991

ABSTRACT: Porphobilinogen synthase (PBGS) is a Zn(II) metalloenzyme which catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA). The nitrogen of the first substrate ends up in the pyrrole ring of product (P-side ALA); by contrast, the nitrogen of the second substrate molecule remains an amino group (A-side ALA). A reactive mimic of the substrate molecules, 5-chlorolevulinate (5-CLA), has been prepared and used as an active site directed irreversible inhibitor of PBGS. Native octameric PBGS binds eight substrate molecules and eight Zn(II) ions, with two types of sites for each ligand. As originally demonstrated by Seehra and Jordan [(1981) *Eur. J. Biochem. 113*, 435–446], 5-CLA inactivates the enzyme at the site where one of the two substrate molecules binds, and modification at four sites per octamer (one per active site) affords near-total inactivation. Here we report that 5-CLA-modified PBGS (5-CLA-PBGS) can bind up to four substrate molecules and four Zn(II) ions. Contrary to the conclusion of Seehra and Jordan, we find that the preferential site of 5-CLA inactivation is the A-side ALA binding site. On the basis of the dissociation constants, the metal ion binding sites lost upon 5-CLA modification are assigned to the four catalytic Zn(II) sites. 5-CLA-PBGS is shown to be modified at cysteine-223 on half of the subunits. We conclude that cysteine-223 is near the amino group of A-side ALA and propose that this cysteine is a ligand to the catalytic Zn(II). The vacant substrate binding site on 5-CLA-PBGS is that of P-side ALA. We have used ^{13}C and ^{15}N NMR to view [4-^{13}C]ALA and [^{15}N]ALA bound to 5-CLA-PBGS. The NMR results are nearly identical to those obtained previously for the enzyme-bound P-side Schiff base intermediate [Jaffe et al. (1990) *Biochemistry 29*, 8345–8350]. It appears that, in the absence of the catalytic Zn(II), 5-CLA-PBGS does not catalyze the condensation of the amino group of the P-side Schiff base intermediate with the C_4 carbonyl derived from 5-CLA. On this basis we propose that Zn(II) plays an essential role in formation of the first bond between the two substrate molecules.

Porphobilinogen synthase (PBGS)[1] catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA) to form porphobilinogen (PBG), the monopyrrole precursor of porphyrin, chlorophyll, and other naturally occurring tetrapyrroles (Shemin & Russell, 1953). PBGS is a homo-octameric protein with four active sites, four catalytic Zn(II), and four additional Zn(II). Each active site contains two ALA binding sites. The two substrate molecules (ALA) are of course identical; they are differentiated for convenience in terms of their ultimate location in PBG as A-side ALA and P-side ALA.[2] This is illustrated in Figure 1. The enzyme-catalyzed reaction includes formation of a substrate–enzyme Schiff base between C_4 of P-side ALA and an active-site lysine (Nandi & Shemin, 1968; Jordan & Seehra, 1980). The stereochemistry and protonation states of the

† This work was supported by National Institutes of Health Grants ES03654 and RR01224 (E.K.J.) and CA06927 and RR05539 (Institute for Cancer Research) and by an appropriation from the Commonwealth of Pennsylvania.

* Author to whom correspondence should be addressed at her present address: Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111.

[1] Abbreviations: ALA, 5-aminolevulinate; βME, 2-mercaptoethanol; 5-CLA, 5-chlorolevulinate; 5-CLA-PBGS, 5-CLA-modified PBGS; MMTS, methyl methanethiosulfonate; PBG, porphobilinogen; PBGS, porphobilinogen synthase.

[2] A-side ALA is the substrate which contributes the amino group and the acetyl side chain to PBG. P-side ALA is the substrate which contributes the pyrrole nitrogen and the propionyl side chain to PBG.

0006-2960/92/0431-2113$03.00/0