ATP-Dependent Human Erythrocyte Glutathione-Conjugate Transporter. I. Purification, Photoaffinity Labeling, and Kinetic Characteristics of ATPase Activity†

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Received August 27, 1997; Revised Manuscript Received February 12, 1998

ABSTRACT: Dinitrophenyl *S*-glutathione (DNP-SG) ATPase is a 38 kDa membrane protein expressed in erythrocytes and other tissues. Although stimulation of ATP hydrolysis catalyzed by DNP-SG ATPase has been demonstrated in the presence of several structurally unrelated amphiphilic ions, structural and functional properties of this protein have not been well-defined. In the present study, we have developed an improved protocol for the purification of DNP-SG ATPase and investigated its kinetic and substrate-binding properties. The purification procedure was based on highly specific elution of the 38 kDa protein from DNP-SG affinity resin in the presence of ATP. The protein could not be eluted using either ADP or adenosine-5′-[β,γ-methylene]triphosphate (methylene-ATP), a nonhydrolyzable analogue of ATP. Doxorubicin (DOX), a weakly basic anthracycline chemotherapy agent, was found to be the preferred activator for stimulation of ATP hydrolysis by the enzyme. ATP binding to the enzyme was demonstrated using 8-azido-ATP photoaffinity labeling and binding of trinitrophenyl (TNP)-ATP, a fluorescent analogue of ATP. The photoaffinity labeling of DNP-SG ATPase (38 kDa) was saturable with respect to 8-azido ATP (K_d = 2 μM), indicating that the enzyme was capable of specific and saturable binding to ATP. DNP-SG binding was evident from the purification procedure itself and was also demonstrable by quenching of tryptophan fluorescence. Results of quenching of tryptophan fluorescence as well as radioactive isotope-binding studies indicated that DOX was bound to the purified protein as well.

Integrity of the biconcave disk shaped membrane of erythrocytes is essential for the gas-exchange functions of erythrocytes. Structural integrity and deformability of the erythrocyte membrane and membrane skeleton decrease with age due to accumulating structural lesions. Erythrocytes possess effective defense mechanisms for retarding membrane damage. Because membranes are particularly susceptible to lipophilic or amphiphilic oxidants and electrophiles, antioxidant defenses are prominent among these mechanisms. Erythrocytes contain a relatively high concentration of GSH, a sulfhydryl containing nucleophilic tripeptide which can protect cells from electrophilic stress (*1*, *2*). GS-E[1] are important intermediates in the metabolism of electrophiles. Although these are generally less reactive than the parent electrophile, increased electrophilic or free-radical reactivity can occur (*3*−*5*). GS-E can also inhibit critical GSH linked enzymes such as glutathione reductase and glutathione S-transferase (*6*, *7*). Because erythrocytes lack the complete battery of mercapturic acid pathway enzymes, their principal defense against the potential toxicities of GS-E is the extrusion of these conjugates by ATP-dependent and ATP-independent membrane transport mechanisms.

The ATPase activity of DNP-SG ATPase, a 38 kDa membrane protein, has been shown to be stimulated by of GS-E (*8*), conjugated bilirubins, bile acids (*9*), and the classical P-glycoprotein substrates such as doxorubicin (*10*). Its presence has been demonstrated in several human tissues including lung, muscle, liver, and pancreas (*9*−*13*). During previous studies of ATP-dependent transport of doxorubicin (DOX) across erythrocyte membranes vesicles, we observed increased ATP-dependent uptake of DOX into vesicles that were enriched in DNP-SG ATPase through incorporation of the purified enzyme; this uptake was competitively inhibited by GS-E such as the GSH-conjugates of ethacrynic acid (EA-SG) and 1-chloro-2,4-dinitrobenzene (DNP-SG) (*10*). These studies suggested that DNP-SG ATPase was a unique

† These studies were supported in part by USPHS Grants CA63660 (S.A.), GM32304 (Y.C.A.), CA55589 (S.V.S.), and VA Merit Review grant (P.Z.).

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[1] Abbreviations: GSH, glutathione; GS-E, glutathione electrophile conjugates; DNP-SG, dinitrophenyl *S*-glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; 2ME, 2-mercaptoethanol; PMSF, phenylmethanesulfonylfluoride; BHT, *tert*-butylhydroxytoluene; $C_{12}E_9$, polidocanol; DOX, doxorubicin; G3PD, glyceraldehyde-3-phosphate dehydrogenase; MRP, multidrug-resistance associated protein; P-gp, P-glycoprotein (*mdr*-1 gene product).

S0006-2960(97)02130-2 CCC: $15.00
Published on Web 03/26/1998

transporter, widely distributed in tissues, and able to mediate ATP-dependent transport of amphiphilic organic ions. In this communication, we report a single-step affinity purification of DNP-SG ATPase from erythrocyte membrane and substrate-binding and kinetic properties of highly purified DNP-SG ATPase. In the accompanying communication, we report the reconstitution of purified enzyme in proteoliposomes capable of ATP-dependent transport of DNP-SG and DOX.

EXPERIMENTAL PROCEDURES

Materials. Blood was collected from normal human volunteers according to a protocol approved by the Institutional Review Board. GSH, CDNB, 2ME, PMSF, BHT, EGTA, EDTA, cholesterol, $C_{12}E_9$, ATP, ADP, and horseradish-peroxidase-coupled goat-anti-rabbit antibodies were purchased form Sigma (St. Louis, MO). Adenosine-5′-[methylene]triphosphate tetralithium salt (methylene-ATP) was purchased from Fluka Chemie AG (Buchs, Switzerland). SM-2 biobeads, SDS, and reagents for SDS−PAGE were purchased from Bio-Rad Laboratories (Hercules, CA). DOX (Adriamycin) was obtained from Adria Laboratories (Columbus, OH). [14-^{14}C]DOX (specific activity 57 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). [Glycine-2-^{3}H]GSH (specific activity 43.8 Ci/mmol) and [γ-^{32}P]ATP (specific activity 10 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [γ-^{32}P]8-Azido-ATP (specific activity 9.5 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Rabbit anti-human antibodies toward the 38 kDa human erythrocyte DNP-SG ATPase protein as well as polyclonal antibodies toward human G3PD used in these studies were the same as described previously (*11*, *12*). Monoclonal anti-human glycophorin antibodies were obtained from Sigma (St. Louis). Monoclonal antibodies specific for the 35 and 67 kDa domains of human erythrocyte band III protein were a generous gift from Professor Michael Jennings at the University of Arkansas for Medical Sciences, Little Rock. Monoclonal antibodies against multidrug-resistance-associated protein (MRP) were kindly provided by Dr. Guido Zaman, The Netherlands Cancer Institute, Amsterdam.

Determination of DNP-SG ATPase Activity. DNP-SG ATPase activity was measured by organic extraction of hydrolyzed γ-phosphate using ammonium molybdate according to the method of Knowles and Leng (*14*) with slight modifications as described by us previously (*11*). Background ATP hydrolysis was estimated by quantifying γ-phosphate liberated from ATP in the absence of protein. This background was subtracted from hydrolyzed γ-phosphate observed in the presence of protein and activator (stimulated ATPase activity) and in the presence of protein without activator (unstimulated ATPase activity). Specific activator stimulated ATPase activities presented were obtained by subtracting unstimulated ATPase activity from stimulated ATPase activity. Protein was determined by the dye binding assay described by Minamide and Bamburg (15).

Purification of DNP-SG ATPase from Human Erythrocytes. All purification steps were carried out at 4 °C unless otherwise specified. Water and phosphate buffers were passed over Chelex-100 (Bio-Rad, Richmond, VA) resin to minimize metal contamination. All buffers were prepared fresh and filter sterilized. Except for the blood collection buffer, all buffers used in purification contained 1.4 mM 2-ME, 0.1 mM PMSF, 0.1 mM EDTA, and 50 μM BHT in addition to other constituents. The blood collection buffer consisted of 200 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM PMSF, 28 mM 2-ME, and 1 mM BHT. This buffer was diluted 1:1 with 1000 units/mL heparin prior to use, and 2 mL was drawn into the syringe for every 20 mL blood to be collected. For each purification, 20−60 mL of blood was collected by venipuncture from human volunteers. The plasma fraction and buffy-coat were collected and discarded after centrifugation at 1300*g* in a Sorvall RC5C refrigerated centrifuge. The erythrocytes were washed with several volumes of balanced salt solution, pH 7.4, containing 138 mM NaCl, 5 mM KCl, 0.3 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4 mM $NaHCO_3$, and 5.6 mM glucose. Washed erythrocytes were lysed in 10 mM Tris-HCl, pH 7.4, and ghosts obtained by centrifugation at 28000*g* were washed repeatedly with the same buffer to remove detectable residual hemoglobin. DNP-SG coupled to CNBr-activated Sepharose 4B resin was then added to the washed ghosts and sonicated on ice in 15 s segments at 50 W for a total of 2 min. The mixture was incubated overnight at 4 °C on a shaker, after which the mixture was sonicated again for 1 min. The resin (bed volume approximately 3 mL) was washed 20 times by diluting the bed volume of the ghost−resin mixture with washing buffer [10 mM EDTA, 150 mM NaCl, and 0.01% (w/v) SDS in 10 mM Tris-HCl, pH 7.4] followed by vortexing and centrifugation at 5000*g* for 10 min. Subsequently, the resin was washed twice with 40 mL washing buffer containing 10 mM sodium acetate, and twice with washing buffer containing 2 mM ADP. The resin was then equilibrated with the lysis buffer. DNP-SG ATPase was eluted by incubating the resin for 1 h at 37 °C with a solution containing 10 mM Tris-HCl, pH 7.4, 10 mM ATP, 10 mM $MgCl_2$, and 0.01% SDS. The eluate was concentrated using the Amicon Centriprep concentrator, followed by sequential dialysis against lysis buffer containing 2% DE-52 and lysis buffer containing 1% (w/v) Chelex resin for 24 h each with two buffer exchanges of 1 L each. $C_{12}E_9$ (0.025%) was added to the final purified protein.

8-Azido ATP Photoaffinity Labeling of DNP-SG ATPase. Photoaffinity labeling with 8-azido ATP was performed using appropriate controls as described by us previously (*16*). Purified DNP-SG ATPase (0.05 μg) was incubated with 20 μM 8-azido ATP and irradiated for 90 s using a short-wavelength hand-held UV lamp resting on open tubes. Immediately after irradiation, SDS−PAGE sample buffer containing SDS and 2-ME was added and the reaction mixtures were incubated for 30 min at 37 °C prior to SDS−PAGE. After thorough washing, radioactivity in gels was visualized by autoradiography and quantified using an ImageQuant phosphorimager.

Quenching of Tryptophan Steady-State Fluorescence of DNP-SG ATPase. Fluorescence-quenching studies were performed according to the procedure described by Eftink and Ghiron (*17*). The fluorescence emission spectra of DNP-SG ATPase were measured using a Hitachi F-4500 fluorescence spectrophotometer. Measurements were performed at 23 °C in a total volume of 1 mL of lysis buffer containing 0.5−1.0 μg/mL protein. The samples were excited at 280 nm with a 5 nm excitation slit, and emission spectra were

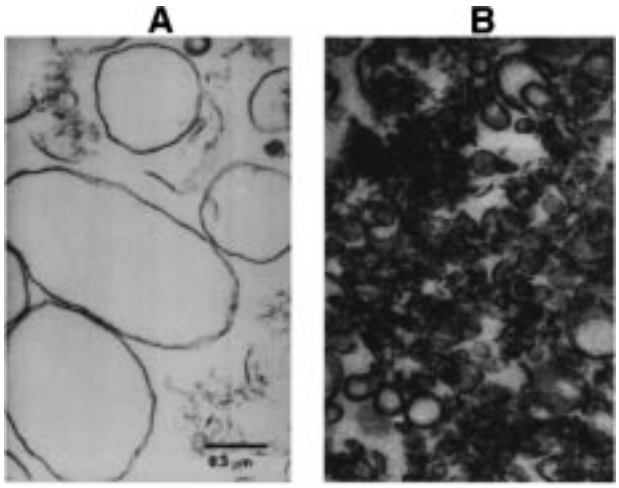

FIGURE 1: The effect of EDTA and NaCl on erythrocyte membrane vesicles. Erythrocyte membrane vesicles were prepared by sonication of washed ghosts. Electron micrographs of these vesicles before (panel A) and after (panel B) treatment with wash buffer containing 10 mM EDTA, 150 mM NaCl and 0.01% SDS are presented (magnification 42625×).

recorded between 295 and 425 nm (emission slit 20 nm). The scanning speed was 60 nm/min, and PMT voltage was 950 V. The value of fluorescence intensity at the emission peak (335 nm) after blank subtraction was used for calculations. Stock solutions of ATP and ADP were prepared in 10 mM Tris-HCl buffer, and pH was adjusted to 7.4 with NaOH. Quenching of fluorescence was performed in the presence of 0−100 μM ATP, ADP, DNP-SG, and DOX. Quenching was analyzed by the Stern−Volmer equation: $F_o/F = [Q]K_{SV} + 1$, where F_o is the measured fluorescence intensity without quencher added, F is the fluorescence intensity in the presence of quencher, [Q] is the concentration of a collisional quencher, and K_{SV} is the Stern−Volmer constant. Using the equation $K_{sv} = \tau k_q$ (where τ is the unquenched lifetime of the flurophore and K_q is the observed rate constant for quenching), the K_q can be estimated assuming that observed fluorescence is that of a single tryptophan residue with τ near 5 ns.

Measurement of TNP-ATP binding to DNP-SG ATPase by Fluorescence Spectroscopy. Binding of TNP-ATP was studied using methods described by Devita et. al. (*18*) as used by us previously (*16*). TNP-ATP stock (6.4 mM) was

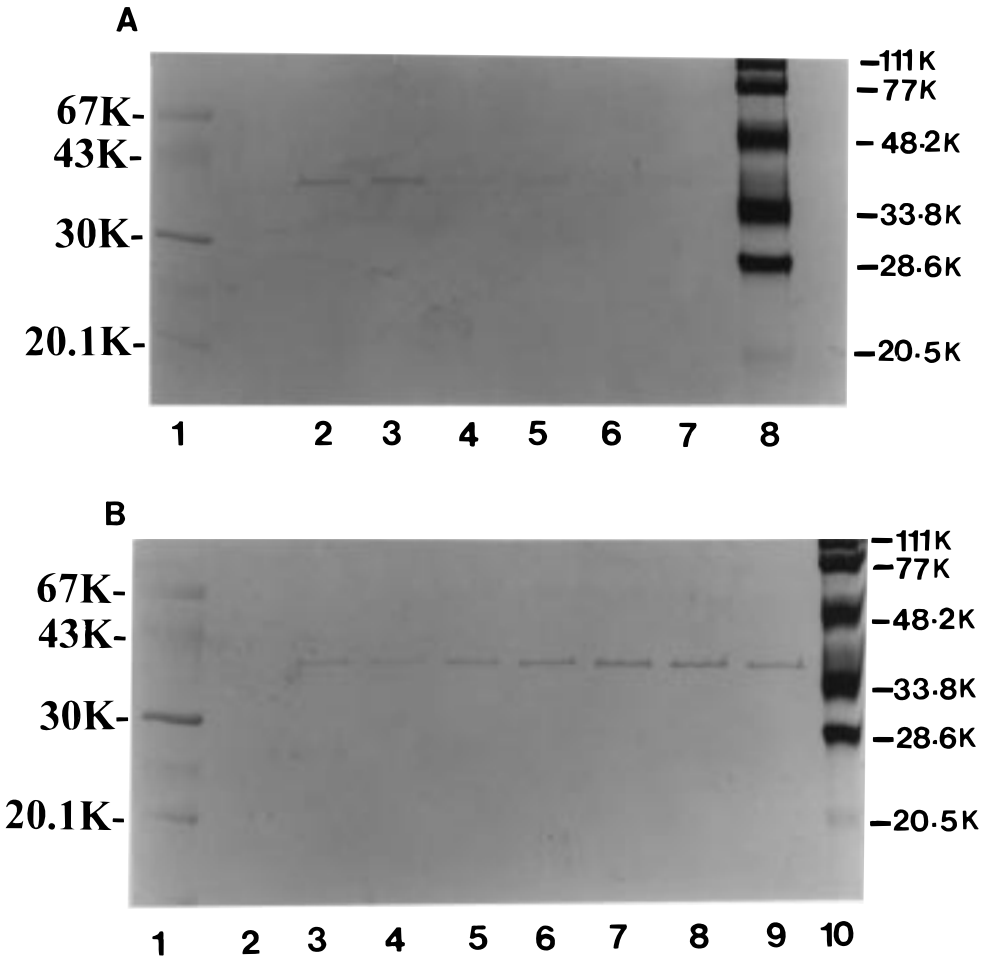

FIGURE 2: Elution of DNP-SG ATPase from the DNP-SG affinity resin. For these studies, purification was performed from 60 mL of blood. Aliquots (5% of total) of washed affinity resin bound to erythrocyte membrane proteins were eluted under varying conditions. The eluted protein (~1 μg) was subjected to SDS−PAGE and silver stained. Standard M_r markers (Pharmacia Biotech) are shown in lane 1 of panels A and B. Lane 8 in panel A and lane 10 in panel B contained low M_r prestained markers (Bio-Rad). Protein eluted in the presence of 10 mM ATP and 10 mM $MgCl_2$ at 4 and 37 °C was loaded in lanes 2 and 3, respectively (panel A). Protein eluted in the presence of 10 mM ATP without $MgCl_2$ at 4 and 37 °C was loaded in lanes 4 and 5, respectively (panel A). Protein eluted in the presence of 10 mM methylene-ATP and 10 mM $MgCl_2$ at 4 and 37 °C was loaded in lanes 6 and 7, respectively (panel A). The time dependence of elution in the presence of Mg^{2+}ATP was evaluated at 4 and 37 °C for 5 min (panel B, lanes 2 and 3, respectively), 10 min (panel B, lanes 4 and 5, respectively), 20 min (panel B, lanes 6 and 7, respectively) and 30 min (panel B, lanes 8 and 9, respectively).

used directly from the vial, as supplied by manufacturer (Molecular Probes, Inc., Eugene OR). There was no change in pH after addition of TNP-ATP to the assay medium up to the concentration of 100 μM nucleotide. The concentration of TNP-ATP added to protein samples ranged from 0 to 30 μM. The samples were excited at 412 nm, and emission spectra were recorded between 500 and 600 nm at 240 nm/min with PMT voltage at 950 V. The values of fluorescence intensity of the emission peak at 535 nm after blank subtraction were used in calculations. In experiments to characterize competition by ATP, fluorescence enhancement was measured in the presence of 9.6 μM TNP-ATP and increasing concentrations of ATP (0−50 μM).

Binding of DOX and DNP-SG to DNP-SG ATPase. The stock solution of 14-[^{14}C]DOX used for these studies contained 36 μM DOX (specific activity 7.5 × 10^4 cpm/nmol). Stock DNP-SG was prepared as a 5 mM solution in water. Purified DNP-SG ATPase was concentrated 10-fold by Amicon-filtration, followed by dialysis. Aliquots of protein containing 3 μg of DNP-SG ATPase were diluted to a final volume of buffer containing 0.72−7.2 μM 14-[^{14}C]-DOX without or with 0.5 mM DNP-SG. DNP-SG was incubated for 60 min at room temperature. Protein was precipitated with 10% TCA, and radioactivity was determined in the supernatant. The pellet was washed and resuspended in scintillant to determine bound radioactivity.

RESULTS

Purification of DNP-SG ATPase. The DNP-SG stimulated ATPase activity in human erythrocyte membrane was first identified by us during studies to delineate the mechanisms responsible for energy dependent efflux of DNP-SG from erythrocytes (*8*). The 38 kDa protein displaying DNP-SG-stimulated ATPase activity was isolated with a relatively poor yield and significant inactivation (*9*−*12*). Present studies were initiated to develop an alternate purification scheme which could offer higher yield of active enzyme to allow more detailed studies of its kinetic and molecular nature. The DNP-SG affinity chromatography step was a logical choice for modifications aimed at improving selectivity and yield. In designing the current purification scheme, we also took advantage of the observations that the 38 kDa protein could be specifically eluted by incubation of the affinity resin with Mg^{2+}ATP but not ADP. These results were consistent with our previous observations of the elution of the rat liver canalicular MOAT from DNP-SG affinity resin by Mg^{2+}ATP (*16*, *19*). The current purification protocol was designed to exploit these observations in order to obtain a larger quantity of sufficiently pure and stable protein for kinetic studies.

On the basis of observations that high concentration of EDTA caused irreversible fragmentation of erythrocyte ghosts (Figure 1), we chose to remove the membrane lipids and proteins from the affinity resin-erythrocyte vesicle mixture by washing it with buffer containing 10 mM EDTA and detergent (0.01% SDS). With repeated washing, we observed a gradual reduction in the pellet bed volume (containing vesicles and affinity resin) to approximately that of the affinity resin alone. The affinity resin was eluted by incubation for 30 min in buffer containing ATP (with or without $MgCl_2$). SDS−PAGE of the supernatant fraction indicated that presence of Mg^{2+}ATP resulted in elution of a

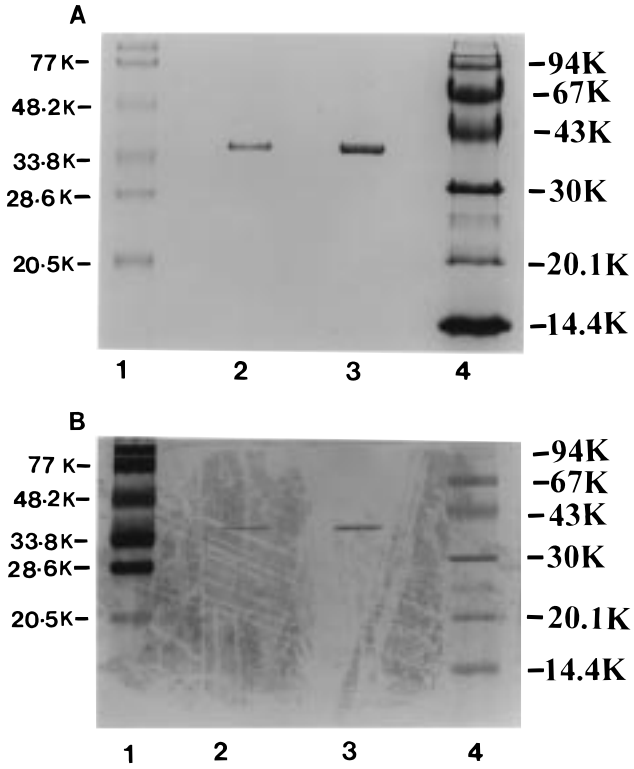

FIGURE 3: SDS−PAGE of purified human erythrocyte DNP-SG ATPase. In both panels A and B, lane 1 contained low-range prestained M_r markers and lane 4 contained unstained standard M_r markers. In panel A (Coomassie brilliant blue stained), 4 and 10 μg of purified DNP-SG ATPase was loaded in lanes 2 and 3, respectively. In panel B (silver-stained), 1 and 2 μg of purified DNP-SG ATPase was loaded in lanes 2 and 3, respectively.

38 kDa protein which eluted much less efficiently without magnesium and did not elute in the presence of methylene-ATP (Figure 2A). The inability of either ADP (used to wash the affinity resin) or the nonhydrolyzable analogue to elute the protein strongly suggested that ATP-hydrolysis was necessary for elution. Qualitatively, elution appeared to be more efficient at 37 °C than at 4 °C (Figure 2A). Time dependence of elution was evaluated at 4 and 37 °C by removing aliquots of ATP-containing elution buffer after 5, 10, 20, and 30 min (Figure 2B). These studies showed that elution was essentially complete within 30 min and that a small difference in the rate of elution between 4 and 37 °C was qualitatively apparent at 5 min. The final preparation of DNP-SG ATPase showed a single band in SDS gels stained by either silver (Figure 3A) or Coomassie brilliant blue (Figure 3B) and the intensity of the bands were proportional to the amounts loaded on gels. Taken together, these results indicated that a DNP-SG binding erythrocyte membrane protein with apparent M_r of 38 kDa could be eluted from a DNP-SG affinity resin by the hydrolysis of ATP.

Immunologic Characterization of DNP-SG ATPase. During early purification attempts, we observed variable contamination of the purified DNP-SG ATPase with other RBC membrane proteins including glyceraldehyde-3-phosphate dehydrogenase (G-3PD), band III fragments, and particularly glycophorins. Some of these proteins may bind specifically to DNP-SG affinity matrix. For example, it has been shown that the Rossmann fold of G-3PD recognizes glutathione conjugates (*20*). Nevertheless, sequential washings of the

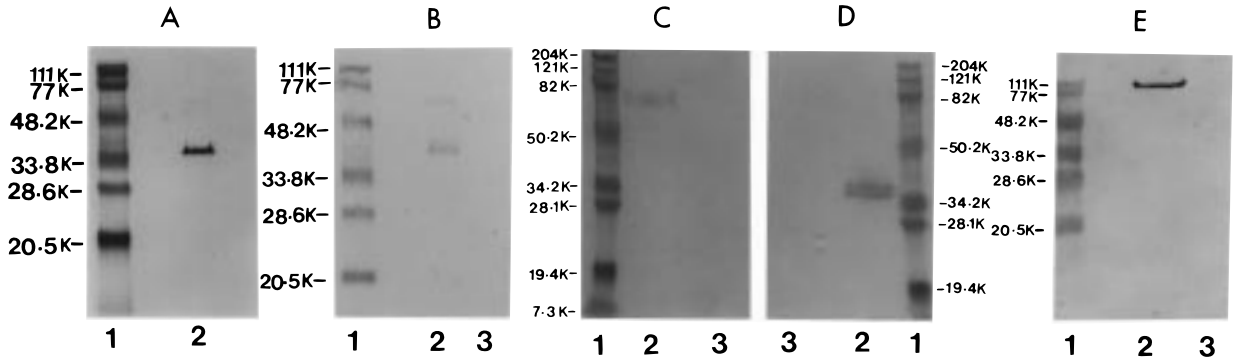

FIGURE 4: Immunologic characterization of DNP-SG ATPase. DNP-SG ATPase (2 μg) was subjected to SDS−PAGE and transblotted to nitrocellulose membranes. Sources of the polyclonal rabbit-anti-human DNP-SG ATPase (panel A), polyclonal rabbit-anti-human G3PD (panel B), monoclonal mouse-anti-human band III (panel C), monoclonal mouse-anti-human glycophorin (panel D), and monoclonal mouse-anti-MRP antibodies (panel E) are described in the Experimental Procedures. Goat-anti-rabbit horseradish peroxidase linked secondary antibodies and 4-chloro-1-naphthol were used to develop the western blots against DNP-SG ATPase and G3PD. Alkaline phosphatase linked secondary antibody and *p*-nitrobluetetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were used to develop the Western blots against band III, glycophorin and MRP. In panels A, B, and E, lane 1 contained low-range prestained M_r markers; in panels C and D, lane 1 contained broad range prestained markers. Lane 2 in panel A and lane 3 in panels B−E contained 2 μg of purified DNP-SG ATPase. In panels B−D, lane 2 contained SDS extracts of human erythrocyte ghosts as positive controls for G3PD, band III, and glycophorin, respectively. In panel E, lane 2 contained SDS extract of human small cell lung cancer cell line membrane fraction as a positive control for MRP.

Table 1: Purification of DNP-SG ATPase from Human Erythrocytes[a]

fraction	activator stimulated activity[b] (milliunits) DNP-SG	DOX	protein (mg)	specific activity (milliunits/mg of protein) DNP-SG	DOX	yield (%) DNP-SG	DOX	purification fold DNP-SG	DOX
vesicles	48.0	44.0	63.6	0.76	0.69	100	100		
			DNP-SG Affinity Chromatography[c]						
unabsorbed	5.4	4.6	56.6	0.095	0.081	11.3	10.5		
eluate	15.7	19.4	0.096	163.5	202.1	32.7	44.2	221	298

[a] Sixty milliliters of blood was used for purification. [b] One milliunit of activity was defined as 1 nmol of ATP hydrolyzed/min at 37 °C. Activator stimulated ATPase activities presented were calculated by subtracting basal (unstimulated) ATPase activities from activities obtained in the presence of the activator, either 120 μM DNP-SG or 10 μM DOX. [c] DNP-SG affinity chromatography was performed as described in Experimental Procedures.

affinity resin as described in Experimental Procedures rendered these potential contaminants undetectable in Western-blot analyses using antibodies specific for G-3PD, for the 35 and 67 kDa fragments of band III, and for glycophorins. The purified protein was recognized by polyclonal antibodies raised against previously purified DNP-SG ATPase (*12*), but not by monoclonal antibodies against MRP (Figure 4).

ATPase Activity of DNP-SG ATPase. The purification of DNP-SG ATPase was monitored by measuring basal (in the absence of activator) as well as DOX- or DNP-SG-stimulated ATPase activity. To prevent interference by ATP in the determination of ATPase activity, the purified protein was dialyzed against buffer containing 2% (w/v) DE-52 at pH 7.4. Basal ATPase activity of the purifed protein (126 ± 24 nmol/min/mg of protein, $n = 6$ separate purifications with each determination in triplicate) was stimulated an average of 2.5- and 2.1-fold (311 ± 47 and 264 ± 56 nmol/min/mg of protein, $n = 6$) by 120 μM DNP-SG and 10 μM DOX, respectively. Results of a representative purification expressed in terms of DNP-SG- and DOX-stimulated ATPase activity are presented in Table 1. DNP-SG affinity purification resulted in approximately 200-fold purification of enzyme activity, a result an order of magnitude improved over that obtainable by the previous purification method (*10*−*12*). ATPase activity of the purified protein was stimulated in the presence of several other compounds in addition to DNP-SG (Table 2). Although the protein was purified by affinity chromatography using an anionic ligand, DNP-SG, the efficiency of stimulation of ATP-hydrolysis

Table 2: Stimulation of DNP-SG ATPase Activity by Various Activators[a]

activators	S_{50}[b] (μM)	V_{max} (milliunits/mg of protein)	k_{cat} (s^{-1})	k_{cat}/S_{50} (s^{-1} M^{-1})
CH_3-SG	137	148	0.0937	684
H_3C-$(CH_2)_9$-SG	1528	469	0.2970	194
ESA-SG[c]	674	337	0.2134	317
EA-SG[c]	74	258	0.1634	2208
DNP-SG[c]	135	416	0.2635	1952
bilirubin ditaurate	103	452	0.2863	2780
doxorubicin	3.3	230	0.1457	44 152

[a] One milliunit of enzyme catalyzed the hydrolysis of 1 nmol of ATP/min at 37 °C. Basal (unstimulated) ATPase activity was subtracted from activity observed in the presence of activator to obtain stimulated ATPase activity. [b] S_{50}, the concentration of activator yielding half-maximal stimulation, and V_{max} were obtained by nonlinear regression fit of the equation $v = V_{max}/(1 + S_{50}/[\text{activator}])$ to the data obtained from three measurements at five concentrations of substrate. The r^2 values obtained from regression were ≥0.97. [c] Glutathione conjugates of 9,10-epoxy-stearic acid, ethacrynic acid ([2,3-dichloro-4-(2-methylenebutryl)phenoxy]acetic acid), and 1-chloro-2,4-dinitrobenzene, respectively. [d] k_{cat} was calculated assuming $M_r = 38\,000$.

(K_{cat}/S_{50}) was greater for the weakly cationic anthracycline, DOX. Relatively high activity was also observed with the anionic compounds bilirubin ditaurate and EA-SG. *S*-Alkyl derivatives of glutathione and the glutathione conjugate of epoxy stearic acid were relatively poor activators. After DOX, the lowest S_{50} (concentration of activator causing half-maximal stimulation) values were observed for the glu-

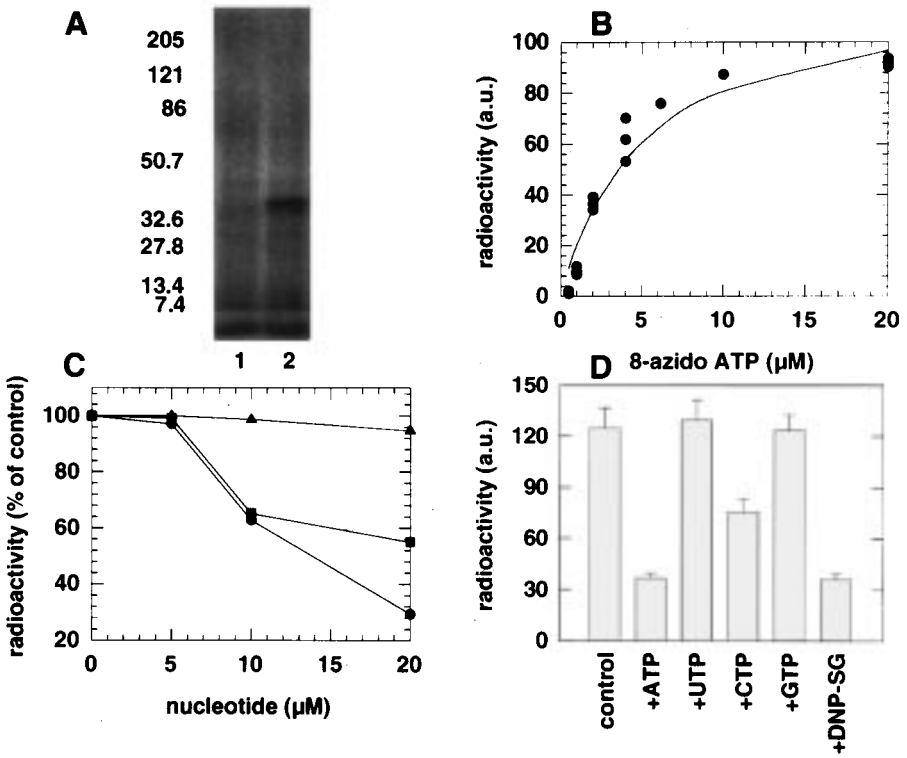

FIGURE 5: 8-Azido ATP photoaffinity labeling of DNP-SG ATPase. Purified DNP-SG ATPase (0.05 μg) was incubated with 20 μM 8-azido ATP and irradiated for 90 s by placing a hand-held UV lamp on top of open glass tubes. This was followed by addition of SDS−PAGE sample buffer containing SDS and 2-ME. After thorough washing, radioactivity in gels was visualized and quantified using an Image-Quant phosphorimager. In panel A, lane 1 contained an unirradiated solution of DNP-SG ATPase and 8-azido-ATP and lane 2 contained the corresponding irradiated sample. Results of additional binding and competition studies are presented in the form of quantified radioactivity by phosphorimager (panels B−D). The effect of increasing concentrations of 8-azido ATP on photoaffinity labeling at 38 kDa is shown in panel B. The effect of increasing concentrations of ATP (●), TNP-ATP (■), and ADP (▲) on radiolabeling at 38 kDa are shown in panel C. The effect of other nucleotides and DNP-SG on photoaffinity labeling by 8-azido-ATP are presented in panel D.

tathione conjugates of compounds with aromatic substituents, CDNB, and ethacrynic acid. The GSH conjugates with bulky alkyl groups (*S*-*n*-decyl-glutathione and *S*-9,10-epoxystearyl glutathione) displayed relatively higher S_{50} values. These results suggest that ATPase activity was stimulated by interaction of structurally dissimilar compounds, which have in common their amphiphilic nature and similar size.

8-Azido-ATP Labeling of DNP-SG ATPase. The purified DNP-SG ATPase was photoaffinity labeled using 8-azido-[^{32}P]ATP. A radiolabeled band corresponding to the 38 kDa protein was observed after UV irradiation (Figure 5, panel A). Photoaffinity labeling of the 38 kDa protein was saturable with respect to azido-ATP concentration ($K_d = 2.0$ μM), indicating that the protein was capable of specific and saturable ATP binding (Figure 5, panel B). The inhibition of labeling by ATP and TNP-ATP was found to be concentration dependent (Figure 5, panel C). Addition of 20 μM UTP or GTP did not significantly inhibit azido-ATP labeling, but 20 μM CTP reduced labeling to 67% of control (Figure 5, panel D). Addition of 20 μM DNP-SG resulted in reduction of labeling to 32% of control (Figure 5, panel D). This finding was consistent with our previous observations on the effect of DNP-SG on azido-ATP photoaffinity labeling of rat liver MOAT (*16*).

Binding of ATP and TNP-ATP to DNP-SG ATPase. ATP binding was also characterized through competitive binding studies of ATP and TNP-ATP, a fluorescent ATP analogue. The fluorescence of TNP-ATP (excitation at 412 nm, emission at 535 nm) has been shown to increase as a result of binding to ATP-binding sites of proteins (*17*, *19*). In the presence of purified DNP-SG ATPase, the increase in fluorescence intensity of TNP-ATP was found to be depend-

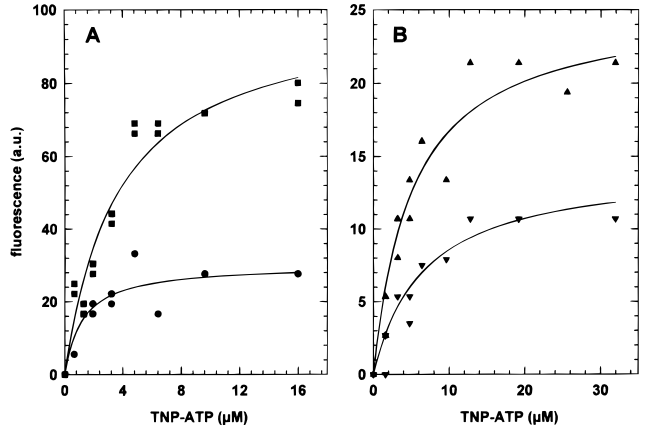

FIGURE 6: TNP-ATP binding to DNP-SG ATPase. Purified DNP-SG ATPase was suspended in lysis buffer containing 0.025% polidocanol containing varying concentrations of TNP-ATP. Samples were placed in a Hitachi 4500 fluorometer and excited at 412 nm (950 V). Emission spectra were recorded between 500 and 600 nm. The height of the emission peak at 535 nm after subtraction of blank were plotted vs TNP-ATP concentration. Concentration dependence of TNP-ATP binding was studied by varying concentration of TNP-ATP in samples containing purified DNP-SG ATPase at either 5 μg/mL (●) or 20 μg/mL (■) (panel A). The effect of ATP on saturable binding of TNP-ATP was studied at varying concentrations of TNP-ATP without (▲) or with (▼) 50 μM ATP in the presence of 5 μg/mL DNP-SG ATPase (panel B).

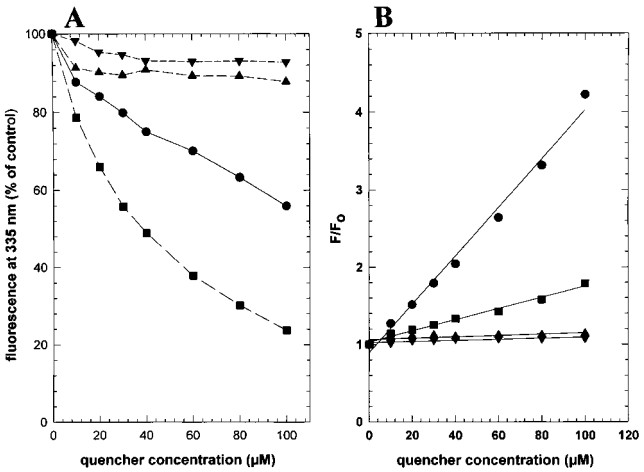

FIGURE 7: Effect of nucleotides or substrates on quenching of tryptophan fluorescence of DNP-SG ATPase. Tryptophan fluorescence emission spectra of 1 μg/mL DNP-SG ATPase were recorded measured as described in Experimental Procedures. Quenching of intrinsic DNP-SG ATPase fluorescence with respect to varying concentrations of DNP-SG (●), DOX (■), ATP (▲), or ADP (▼) are presented (panel A). The ratio of measured fluorescence intensity without (F_o) and with quencher (F) at several concentrations of DNP-SG, DOX, ATP, or ADP were plotted to determine the Stern−Volmer constant (K_{SV}) (panel B).

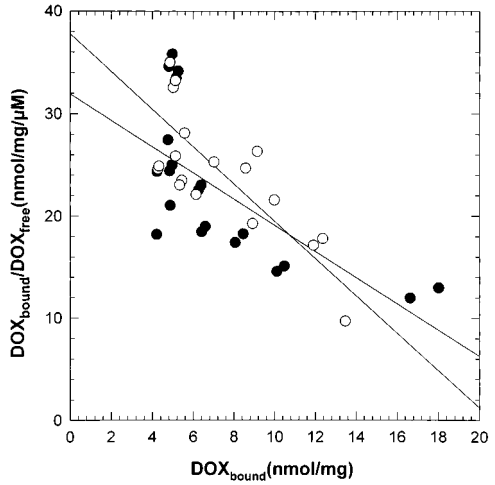

FIGURE 8: Effect of DNP-SG on DOX binding to purified DNP-SG ATPase. Reaction mixtures containing 200 ng of purified DNP-SG ATPase each were incubated in a final volume of 100 μL in the presence of varying concentrations of 14-[^{14}C]DOX between 0.72 and 7.2 μM (sp. act. 7.5×10^4 cpm/nmol units) in the absence (○) or presence (●) of 0.5 mM DNP-SG for 1 h. Protein was precipitated by addition of 10% TCA and incubation for 20 min at 4 °C. The supernatant of 12000*g* centrifugation was collected and radioactivity was determined in the supernatant fractions as well as the pellet which was solubilized in scintillation cocktail.

ent on protein concentrations and saturable with respect to TNP-ATP concentration (Figure 6, panel A). Addition of ATP blunted the maximal increase in TNP-ATP fluorescence in the presence of DNP-SG ATPase (Figure 6, panel B). Results of these studies confirmed the presence of an ATP-binding site on DNP-SG ATPase.

Excitation at 280 nm of solutions containing protein alone revealed significant intrinsic fluorescence with an emission peak at 335 nm. Quenching of intrinsic fluorescence was used to study substrate-binding properties of DNP-SG ATPase. Quenching of intrinsic fluorescence by DNP-SG, DOX, or ATP was found to be saturable with respect to concentration, indicating specific binding to the protein (Figure 7A). The bimolecular rate constants for quenching (K_q) for DNP-SG, DOX, and ATP were estimated from Stern−Volmer plots (Figure 7B) at 2.9, 1.5, 0.4, and 0.2×10^{12} M^{-1} s^{-1}. These values are consistent with the presence of an exposed tryptophan residue (*19*) near the binding sites for DNP-SG, DOX, and ATP. Differing degrees of quenching between DNP-SG, DOX, and ATP may be related to the distance between the binding site and the fluorophore.

The effect of DNP-SG on binding of DOX was studied by assessing the effect of 0.5 mM DNP-SG on binding of 14-[^{14}C]-DOX to purified DNP-SG ATPase. The purified protein was incubated with varying concentrations of radiolabled DOX for 1 h followed by precipitation with TCA. From radioactivity measured in the pellet and supernatant fractions, analysis of binding was performed using Skatchard plots (Figure 8). DOX was bound saturably in the pellet fraction. The effect of DNP-SG at a concentration well above its K_m (57 μM) on the binding of DOX was slight (K_d for DOX 1.83 μM vs 1.23 μM with or without DNP-SG, respectively) and not statistically significant. These results suggested that DNP-SG ATPase possesses distinct binding sites for DOX and DNP-SG and that the affinity of DOX binding may be affected by binding of DNP-SG.

DISCUSSION

The results presented in this communication confirm our previous studies which have indicated the presence in erythrocyte membranes of a 38 kDa ATPase which can be purified on the basis of its high affinity toward DNP-SG (*9*−*12*). Highly active and apparently homogeneous 38 kDa protein was obtained from human erythrocyte membranes through a rigorously tested and standardized protocol. The crude erythrocyte membrane fraction contained 1.06 mg of protein/mL of blood. Considering that 96 μg of DNP-SG ATPase could be isolated from membranes derived from 60 mL of blood, or 1.6 μg/mL blood at a yield of approximately 40% (averaged between the yields measured with DNP-SG and DOX as substrates, Table 1), it can be estimated that DNP-SG ATPase represents approximately 0.4% of total erythrocyte membrane protein. Since human blood typically contains 4×10^9 RBC/mL, approximately 1.0×10^{-10} μg of DNP-SG ATPase is present in a single erythrocyte. Assuming that 38 kDa is an accurate measure of the molecular weight of this protein, each erythrocyte would contain 2.63×10^{-14} μmol of DNP-SG ATPase, approximately 16 000 copies of the protein per cell. For comparison, erythrocyte membrane cytoskeletal proteins which represent the vast majority of membrane proteins are present at 30000−200000 copies/cell. Drug-selected K562 human leukemia cells 600-fold resistant to vincristine, with marked overexpression of P-gp, have been estimated to contain 200000 copies of P-gp/cell (*21*).

Saturable binding of TNP-ATP indicated that DNP-SG ATPase is an ATP-binding protein. Photoaffinity labeling of the 38 kDa protein by 8-azido-ATP confirmed the presence of an ATP-binding site on DNP-SG ATPase. The inhibition of azido-ATP labeling in the presence of DNP-SG was consistent with our previous findings with rat liver canalicular MOAT (*16*). This inhibition may be due to a nonspecific effect of DNP-SG on the efficiency of photo-

affinity labeling or due to decreased affinity for 8-azido-ATP as a result of specific binding of DNP-SG. The latter is more consistent with a model for DNP-SG stimulated ATP hydrolysis in which specific binding of DNP-SG results in a conformational shift which stimulates ATP-hydrolysis. Conformational shifts which result from ATP-hydrolysis are associated with decreased affinity for DNP-SG. Perhaps the best evidence for this model was the affinity purification procedure itself. The enzyme was tightly bound to the affinity resin and could not be eluted by ADP or methylene-ATP, but addition of Mg^{2+}ATP resulted in release of DNP-SG ATPase from the affinity resin.

The studies of intrinsic fluorescence quenching indicated the presence of a fluorescent residue at or near the active site. Differing degrees of quenching of fluorescence by DNP-SG, DOX, and ATP may be due to distinct loci at which each is bound in the vicinity of a fluorophore. A competitive binding assay also indicated that DOX and DNP-SG may be bound at distinct but spatially related sites. Although the binding site for DNP-SG and DOX appeared to be distinct, ATP hydrolysis was stimulated by both compounds as well as by other glutathione conjugates. Although several mono- and dianionic metabolites have been shown to stimulate ATP-hydrolysis by this protein (*9*), the present results confirm our previous findings that the presence of the anionic group(s) is not essential for stimulation of ATPase activity by DNP-SG ATPase. These observations suggest that aromatic groups of lipophilic or amphiphilic compounds may be important for binding and stimulating the ATP-hydrolysis by DNP-SG ATPase. Stimulation of ATPase activity by compounds such as DOX or dinitrophenol, which possess planar lipophilic groups as well as anionic conjugates of hydrophobic compounds with or without planar lipophilic groups, suggests that DNP-SG ATPase may possess a complex binding site with two adjacent domains: one capable of binding lipophilic moieties [with higher affinity binding to lipophilic moieties containing an aromatic group than alkyl-groups] and the other capable of binding anionic groups (with higher affinity for GSH than other anionic ligands). Since neither GSH nor GSSG alone cause significant stimulation of ATPase activity (unpublished observations), we speculate that the presence of at least some lipophilic constituent is necessary to stimulate ATP hydrolysis. In such a model, the structure and size of the lipophilic constituent would be a primary determinant of the ability of a given amphiphile to stimulate ATPase activity.

The stimulation of the ATPase activity of Dnp-SG by organic compounds invites comparisons with other transport proteins, specifically Pgp (*22*, *23*) and MRP (*24*). While an overall similarity in function is evident, examination of transport kinetics and other parameters of the proteins strongly indicates that Dnp-SG ATPase is responsible for transport processes distinct from those catalyzed by Pgp and MRP. The critical test of this assumption would be the reconstitution of functional transport activity of Dnp-SG ATPase in artificial proteoliposomes. We have thus extended the present studies to the reconstitution. The results of this work are described in the following paper in this issue.

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BI972130Z