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Site-specific Crosslinking of Annexin Proteins by 1,4-Benzoquinone: A Novel Crosslinker for the Formation of Protein Dimers and Diverse Protein Conjugates

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Electronic Supplementary Information

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Materials and Methods

Dicyclopentadiene, 1-methoxycyclohex-1,3-diene, 4-azidoaniline hydrochloride, 2-mercaptoethanol, 6-aminohexanol, 2,2'-(ethylenedioxy)diethanediol were purchased form Sigma-Aldrich Inc and used as received. Cyclopentadiene (11) was prepared from the cracking of dicyclopentadiene according to the procedure in *Org. Synth., Coll. Vol. VII* **1990**, 339. Sodium azide, ascorbic acid sodium salt, and 1,4-benzoquinone were purchased from Alfa Aesar. 1,4-Benzoquinone was purified by sublimation, and the solid stored at -20^{o}C, or as a 100 mM stock solution in DMSO at -20 ^{o}C for brief periods. Buffered solutions of rh-annexin V were made up with BDH sodium phosphate salts using HPLC grade water from Honeywell Burdick & Jackson. Working concentrations of rh-annexin V were prepared from 30 μM stock solutions stored at -80 ^{o}C. Unless otherwise specified, all reactions were carried out at 23^{o}C, in 0.1 M sodium phosphate buffer in either 0.5 or 1.5 mL microcentrifuge tubes.

Separations of small molecule fractions after protein reactions were accomplished as described below for the preparation of **(5).** Centrifugation was accomplished using either an Eppendorf Model *5424* Microcentrifuge or an Eppendorf Model 5804 R Centrifuge. Electrospray Mass Spectrometry was performed on a Thermo-Finnegan LCQ Classic Ion Trap Mass Spectrometer: Sheath Gas Flow Rate, 30; Spray Voltage, 3.00 kV; Capillary Temperature, 250 ^{o}C; Capillary Voltage, 46 V. Samples for analysis were prepared by protocols analogous to that described in http://www.millipore.com/techpublications/tech1/tn072 using Varian Omix C4 Pipette Tips (100

μL); , 75% acetonitrile/0.1% trifluoroacetic acid (TFA) was used as a wetting solution; equilibration and washing solution consisted of 0.1% TFA, and the elution solution was comprised of 90 % acetonitrile/0.1 % formic acid. For the latter, elution volume was 100 μL, all of which was directly infused into the LCQ mass spectrometer. The data was generally collected for 3-5 min after infusion at a flow rate of 10 μL/min.

Protein concentrations were determined from the UV absorbance at 280 nm with a Molecular Devices SpectraMax M2 Multi-Mode Microplate Reader using the protein's extinction coefficient generated by ProtParam (http://www.expasy.org/tools/protparam.html). LCMS was performed on a Thermo Scientific Finnegan Surveyor HPLC system connected to an LCQ mass spectrometer. A BioBasic C18 column (100 x 2.1 mm) from Thermo Scientific was used for separations. The solvent system was composed of 0.05% formic acid in water (A) and 0.04% formic acid in acetonitrile (B). The sample was introduced into the column at 0.2 mL/min for 5 min using 98% of solvent A and 2% of solvent B. After the initial "wash" that allowed full buffer removal prior to MS data collection, the sample was analyzed using 25 minutes gradients (from 98%A; 2%B to 2%A; 98%B).

For SDS-PAGE analyses of the reactions of **(5),** samples (desalted by centrifuge filtration and dried using a Savant SpeedVac Concentrator Model 111V), were mixed (1:1 by volume) with SDS sample-loading buffer (Bio-Rad Cat# 161-07379), followed by adding 2-mercaptoethanol as directed, boiling five minutes, and then centrifuging five minutes at 17K X g. The resulting samples were applied to 4-20% TGS pre-cast gels (BioRad), run at 200V. Gels were stained with Coomassie Blue. After destaining, the gels were scanned for analysis. Yields of protein were estimated using the imaging processing program **ImageJ**.

Site-specific Modification of Annexin V by 1,4-Benzoquinone (Formation of AN-S-BQ, (5)). The AN-S-BQ conjugate **(5)** in amounts up 400 μg was prepared according to the following protocol.

Typically, two (micro)centrifuge tubes were prepared in which 180 μL of annexin V from 1 mg/mL protein solutions were diluted into 315 mL of 0.1 M phosphate buffer (PB), pH8.0 and 5 μL of a 100 mM solution of 1,4-benzoquinone in DMSO were then added. The tubes were

placed on a nutator, rocked for 4-5 minutes, and then spun for 30 sec to drain liquid from the cap. In duplicate: a 15 μL aliquot from the bulk volume was reserved for an MS determination and the residual material was transferred to a 4 mL Amicon Ultra centrifugal filter fitted with a 10 kDa cutoff membrane (EMD Millipore UFC801024). The dead volume of the Amicon filters was filled with 0.1 M PB pH 8.0 and then spun at 5000 rpm for 35 minutes at 4 ^{o}C. These spin conditions delivered 4 mL of filtrate and provided the retained protein in 50-100 μL of solvent. The flow through was then discarded, another 4 mL of buffer was added, and the tubes were re-spun. This step was repeated at least two more times, after which each tube generally contained 180-200 μg of **(6**) in 50-100 μL (100 μM **(6)**) of the conjugate. Samples of **(5)** were either used the same day they were prepared, or stored overnight at 4 ^{o}C.

The progress of the reaction was monitored by mass spectrometry (15μL of reaction mixture per injection). For direct protein infusion, a 15 μL aliquot from the reaction mixture was added to 85 μL of 0.1 % formic acid. Residual buffer was removed using C4 Pipette Tips as described above. The protein solution was infused into the LCQ instrument at 10 μL/min with continuous data collection for 5 minutes. Deconvolutions of the raw spectra were performed using ProMass for Finnegan Xcalibur Version 2.5 SR 1 software. LCMS analysis was performed using a 15 μL injection volume into a Thermo Scientific BioBasic-18 column (100 x 2.1mm) connected to a BioBasic 18, 10 x 2.1 mm drop-in guard by the method described above. Raw MS data were deconvoluted using ProMass software. Mass determination of product **(5)** was 35,905 Da, on average for direct infusion, and 35,908 Da on average using LCMS settings. Annexin V appeared at 35,799 Da. The above protocol provides > 95% of singly labeled protein.

Conversion of Annexin V (1) to the Disulfide Annexin-S-S-2-pyridine (6) by 2,2'-Dipyridyl sulfide (7).

Annexin V (**1,** 10 μM) was treated with 2,2'-dipyridyl sulfide **(7,** 3 mM**)** at pH 7 in 0.1 M phosphate buffer for 16 hours. The parent peak of (**5)** in the mass spectrum (35,802 Da) shifts entirely to 35,911 Da, indicating a quantitative reaction of one equivalent of (**7)** to give only the disulfide **(6)**. The small molecule fraction was then separated by centrifuge filtration. Upon treatment of (**6**) at 10 μM, in 0.1 M phosphate buffer, pH 8, for five minutes with 1 mM BQ **(3)**, the peak at 35,911 Da was unchanged. Under these conditions the parent **(1)** would have

completely converted to **(5)**. The differential behavior clearly supports the notion of thiol-specific reactivity of **(1)** with BQ, under said conditions.

Reaction of AN-S-BQ (5) with 2-Mercaptoethanol (9) to give Mono-thiolated (13).

AN-S-BQ (**5**, 10 μM) was treated for ten minutes with 10 mM 2-mercaptoethanol **(8)** in 0.1 M phosphate buffer, pH 8.0 (or 0.1 M PBS, pH 7.4- 8.0). The conjugate **(5)** was completely converted to a product possessing a peak at 35,988 Da corresponding to the addition of a single equivalent of **(8**) to starting substrate **(5)**. Similar specifically mono-thiolated products were observed for 6-mercaptohexanol (**23**) and methyl 3-mercaptopropionate (**24**), with product masses 36,038 Da and 36,030 Da, respectively.

Site-specific Formation of the Crosslinked Heterodimer (19) of Annexin V and Annexin V^{128} (18).

AN-S-BQ **(5)** was incubated with an equimolar amount of annexin V^{128} **(18)** in 0.1 M phosphate buffer, pH 8.0, after separation of the small molecule fraction by centrifuge filtration. The appearance of a peak at 72,179 Da, consistent with the theoretical mass of **(18)**, indicated that the incubation produces annexin-V crosslinked by benzoquinone to annexin V^{128} **(18).** Typically, 10 μM annexin-S-BQ and 10 μM annexin V^{128} in 100 μL of 0.1 M PB at pH 8.0 were incubated overnight for 12-16 hours. The yield of the crosslinked heterodimer is approximately 50%, as shown by SDS-PAGE analysis.

Site-specific Formation of the Homodimer (22) of Annexin V Crosslinked by 2,2'-(Ethylenedioxy)diethanethiol (20).

AN-S-BQ (10 μM) was reacted with 1mM HS-$(CH_2)_2O(CH_2)_2O(CH_2)_2SH$ ((2,2'-ethylenedioxy)diethanethiol, (**20**, HS-PEG2-SH))) in 0.1 M phosphate buffer at pH 8.0, 23^oC, for 1hr. After centrifuge filtration, the quantitative formation of an adduct of mass 36,082 Da, consistent with the theoretical mass of AN-S-BQ-S-PEG-2-SH **(21)** was detected using LCMS. After separation of the small molecule fraction using centrifuge filtration, annexin-S-BQ-S-PEG2-SH **(21)** was incubated with an equimolar amount of AN-S-BQ in 0.1 M phosphate buffer, pH 8.0. at 23^oC, overnight (12-16 hr). A homodimer of mass 71,985 Da, consistent with the theoretical mass of AN-S-BQ-S-PEG2-S-BQ-S-AN **(23)**, was produced in 40-60% yield,

based on analysis by SDS-PAGE.. The product **(22)** was stable in the buffered solvent at room temperature for at least two days.

Reaction of AN-S-BQ (5) with Cyclopentadiene (11).

AN-S-BQ (10 μM) was treated with 20 μL of ~ 20 mM cyclopentadiene **(11)** in MeOH (stock solution stored at-80 C) in a total volume of 100 μL containing 0.1 M PB, at pH 5.5 or 8.0. There was visible precipitation observed upon mixing. The reaction was carried out at 4^{o}C, for 30 minutes or overnight (12-16 hr) and monitored by ESI-MS (direct infusion). As judged by MS peak heights, 30 min runs generally produced less than 50% yield of product, whereas overnight incubations at pH 5.5 resulted in conversions as high as 70%. When the reaction was carried out with concentrations of methanol at 20%, at pH 8.0, conversion to product at 35,972 Da (consistent with the theoretical mass for **(17)**) was essentially quantitative. The product was stable to treatment with 2-mercaptoethanol. Neither annexin V **(1)** nor the thioether **(13)** reacted with **(11).** If **(5)** was pre-treated with excess ascorbic acid, and the reaction carried out as above, no change in the mass spectrum was observed.

Reaction of AN-S-BQ (5) with 1-Methoxycyclohexa-1,3-diene (12).

AN-S-BQ **(5)** (10 μM) was treated with 20 mM 1-methoxycyclohexa-1,3-diene **(12)** in a total volume of 100 μL using 0.1 M PB. The reaction was carried out at 4^{o}C, for 30 minutes and monitored by mass spectrometry (ESI-MS, direct infusion). Yields from 45-60 % as judged by MS peak heights were obtained for product with 36,016 Da, consistent with the theoretical mass for the Diels Alder adduct **(17)**.

Reaction of AN-S-BQ (5) with 4-Azidoaniline Hydrochloride (10).

AN-S-BQ **(5)** at 10 μM, pH 7.0, overnight (12-16 hr) reacts with 1-50 mM of **(10)** to give conversions from 25-100% to a product with 36,039 Da, consistent with the theoretical mass for (**15**, X=N_3). 4-Azidoaniline hydrochloride **(10)** has limited solubility in aqueous media and the condensations occur under heterogeneous conditions over a considerable portion of this concentration range. The reaction can be carried out with 50 mM azidoaniline HCl in water under unbuffered conditions (pH 3-4) with no observable precipitation to give >70% conversions

of **(5)** to the azide. Aniline (10 mM) under these conditions reacts with **(5)** to give modest yields of a 1:1 adduct (<50%)), whereas phenyl azides are unreactive.

Reaction of AN-S-BQ (5) with Sodium Azide (9).

AN-S-BQ **(5)** at 10 μM, in 0.1 M phosphate buffer at pH 5.5, 23^{o}C in the presence of 100 mM sodium azide **(9)** for 30 minutes, gave conversions of 55-60% to a product possessing mass 35,947 Da, consistent with the theoretical mass for the putatively formed **(14)**.

Sedimentation Assay for Liposome Binding.

Small unilamellar vesicles of 1:1 mixtures of DOPS/DOPC (Avanti PolarLipids) were prepared by drying lipid/chloroform solutions under rotary evaporation and suspending in buffer (0.1 M KCl, 50 mM HEPES pH 7.4, 0.1 mM EGTA) to a concentration of 10 mM. The lipid suspensions were extruded through 100-nm pore diameter polycarbonate membranes (EMD Millipore) using an Avestin LiposoFast homogenizer 15-19 times. The final phospholipid concentrations were determined by a phosphate ashing procedure[1]. A sedimentation assay described previously[2] was used to estimate the avidity of phospholipid liposome binding by the protein. Briefly, mixtures of 0.66 μM annexin V samples and 1 mM DOPS /DOPC in the presence or absence of 0.5 mM $CaCl_2$ were incubated for 45 min and then centrifuged through a SPIN-X MWCO 100 K filtering device (Corning) at 3000 x g for 45 min. All samples were prepared in 0.1 M KCl, 50 mM HEPES buffer, pH 7.4, 0.1 mM EGTA and give a final (approximate) free calcium concentration of 0.5 mM. The first filtrate was reserved for analysis. The retentates, which contained the bound protein fraction, were washed once then resuspended in buffer. SDS-PAGE samples were prepared by taking 30 μL of sample and 10 μL of 4X gel sample buffer, boiling five minutes, and centrifuging five minutes at 17K x g. The samples were applied to 4-20% TGS pre-cast gels (BioRad), run at 200V. Gels were stained with Coomassie Blue. After destaining, the gels were scanned for analysis and later dried.

Supplementary References

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