The Affinity of a Major Ca\textsuperscript{2+} Binding Site on GRP78 Is Differentially Enhanced by ADP and ATP\textsuperscript{*}\textsuperscript{3}

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\#3 The abbreviations used are: ER, endoplasmic reticulum; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry.

GRP78 is a major protein regulated by the mammalian endoplasmic reticulum stress response, and up-regulation has been shown to be important in protecting cells from challenge with cytotoxic agents. GRP78 has ATPase activity, acts as a chaperone, and interacts specifically with other proteins, such as caspases, as part of a mechanism regulating apoptosis. GRP78 is also reported to have a possible role as a Ca\textsuperscript{2+} storage protein. In order to understand the potential biological effects of Ca\textsuperscript{2+} and ATP/ADP binding on the biology of GRP78, we have determined its ligand binding properties. We show here for the first time that GRP78 can bind Ca\textsuperscript{2+}, ATP, and ADP, each with a 1:1 stoichiometry, and that the binding of cation and nucleotide is cooperative. These observations do not support the hypothesis that GRP78 is a dynamic Ca\textsuperscript{2+} storage protein. Furthermore, we demonstrate that whereas Mg\textsuperscript{2+} enhances GRP78 binding to ADP and ATP to the same extent, Ca\textsuperscript{2+} shows a differential enhancement. In the presence of Ca\textsuperscript{2+}, the $K_{D}$ for ATP is lowered $\sim$11-fold, and the $K_{D}$ for ADP is lowered around 930-fold. The $K_{D}$ for Ca\textsuperscript{2+} is lowered $\sim$40-fold in the presence of ATP and around 880-fold with ADP. These findings may explain the biological requirement for a nucleotide exchange factor to remove ADP from GRP78. Taken together, our data suggest that the Ca\textsuperscript{2+}-binding property of GRP78 may be part of a signal transduction pathway that modulates complex interactions between GRP78, ATP/ADP, secretory proteins, and caspases, and this ultimately has important consequences for cell viability.

The endoplasmic reticulum (ER)\textsuperscript{3} of eukaryotic cells is the site where secreted proteins and proteins targeted to the plasma membrane and secretory organelles are folded and processed. These proteins are translocated into the ER lumen, where they fold correctly. Correct protein folding is of great importance to the health of the cell, so signaling pathways that monitor the ER environment have developed. These pathways monitor the levels of unfolded or misfolded proteins in the ER lumen and transmit signals to the cytoplasm and nucleus, giving rise to what is termed the ER stress or unfolded protein response (1, 2). Nutritional reporters of an endoplasmic reticulum stress response, and up-regulation has been shown to be important in protecting cells from challenge with cytotoxic agents. GRP78 has ATPase activity, acts as a chaperone, and interacts specifically with other proteins, such as caspases, as part of a mechanism regulating apoptosis. GRP78 is also reported to have a possible role as a Ca\textsuperscript{2+} storage protein. In order to understand the potential biological effects of Ca\textsuperscript{2+} and ATP/ADP binding on the biology of GRP78, we have determined its ligand binding properties. We show here for the first time that GRP78 can bind Ca\textsuperscript{2+}, ATP, and ADP, each with a 1:1 stoichiometry, and that the binding of cation and nucleotide is cooperative. These observations do not support the hypothesis that GRP78 is a dynamic Ca\textsuperscript{2+} storage protein. Furthermore, we demonstrate that whereas Mg\textsuperscript{2+} enhances GRP78 binding to ADP and ATP to the same extent, Ca\textsuperscript{2+} shows a differential enhancement. In the presence of Ca\textsuperscript{2+}, the $K_{D}$ for ATP is lowered $\sim$11-fold, and the $K_{D}$ for ADP is lowered around 930-fold. The $K_{D}$ for Ca\textsuperscript{2+} is lowered $\sim$40-fold in the presence of ATP and around 880-fold with ADP. These findings may explain the biological requirement for a nucleotide exchange factor to remove ADP from GRP78. Taken together, our data suggest that the Ca\textsuperscript{2+}-binding property of GRP78 may be part of a signal transduction pathway that modulates complex interactions between GRP78, ATP/ADP, secretory proteins, and caspases, and this ultimately has important consequences for cell viability.

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Calcium ions are important intracellular messengers that travel from intracellular Ca\textsuperscript{2+} stores (e.g. the ER) and their target proteins located in the cytosol, the mitochondria, or the nucleus (9). It has been proposed that tidal patterns of Ca\textsuperscript{2+} flux in and out of the ER could contribute to the regulation of protein secretion (10). The storage of Ca\textsuperscript{2+} is highly regulated, because high concentrations can be toxic; therefore, cells typically make use of calcium storage proteins such as calsequestrins and calreticulins, which are characterized by high capacity but low affinity for Ca\textsuperscript{2+} ions (11). The biological significance of the Ca\textsuperscript{2+} binding ability associated with GRP78 has been the subject of conjecture. GRP78 has been ascribed a direct and important role in the storage of the rapidly exchanging pool of Ca\textsuperscript{2+} and reported to have a stoichiometry of 1–2 mol of calcium/mol of GRP78 under resting conditions (5), whereas other reports (9) state that GRP78 is able to bind large quantities of Ca\textsuperscript{2+}.

Prolonged ER stress can lead to cell death by inducing the programmed cell death (apoptosis) pathway. Apoptosis plays an essential role in the normal development of multicellular organisms and in the homeostasis of adult tissues (12–14). The pathways leading to apoptosis are controlled by a series of signal transduction cascades, and alterations to these pathways have been implicated in many disease states, including cancer and neurodegenerative conditions (15–17). The mechanism of apoptosis is conserved across species and is mediated by a group of cysteine proteases called caspases (18). Each of these proteases is synthesized as an inactive proenzyme that is proteolytically cleaved to produce the active form. GRP78 forms complexes with caspase-7 and -12 in the ER compartment, implying that this complex formation prevents release of caspase-12 from the ER (19). The complex between GRP78 and caspase-12 is sensitive to the addition of ATP and dATP in a cell extract-based system (19). Deletion of the GRP78 ATP binding domain prevents binding to procaspase-7 and results in loss of the protective effects against etoposide-induced apoptosis. Similarly, a missense mutation in the ATP domain that prevents ATP binding causes loss of ability to protect against ER stress and against etoposide-induced apoptosis (4,
These observations imply that the binding of relatively small effector molecules such as ATP can modulate the protein binding abilities of GRP78. Studies using overexpression or up-regulation of GRP78 or the use of antisense grp78 RNA show that GRP78 can protect against cell death caused by disturbance of ER homeostasis (4, 21).

Although primarily located within the ER lumen, in response to the addition of ER stress inducers to cell cultures, subpopulations of GRP78 have been reported to redistribute to the cytoplasm and the ER membrane (19). However, these data were unable to distinguish between true redistribution or an effect of up-regulation.

Given the previously published data that show that GRP78 interacts with Ca²⁺ and nucleotides (discussed above), we wished to address the hypothesis that GRP78 acts a dynamic Ca²⁺ storage protein and that the binding of Ca²⁺ may be modulated by the binding of nucleotides. To do this, we chose to use a quantitative thermodynamic approach utilizing microcalorimetry. We report here for the first time a quantitative analysis of Ca²⁺, ADP, and ATP binding to wild type and a fully functional and widely used mutant (G565R) (22) of mouse GRP78 and show that Ca²⁺, ADP, and ATP bind cooperatively. Using differential scanning calorimetry (DSC), we show that in the absence of ADP or ATP GRP78 binds Ca²⁺ weakly with a 

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**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals and solvents were purchased from local suppliers and were of AnaLar or greater purity. Column chromatography materials and an AKTA explorer chromatography station were purchased from GE Healthcare.

**Overproduction and Purification of Wild Type and G565R Mutant GRP78**—18 × 500-ml cultures of *Escherichia coli* strain BL21 DE3 harboring plasmid pHis6-Bip:G565R or pHis6-Bip (22–24) were grown in Luria broth supplemented with 100 μg ml⁻¹ ampicillin in 2-liter baffled glass Erhrlenmeyer flasks at 37 °C in an orbital incubator set at 160 rpm. At midlog growth phase, the cultures were made 0.2 mg ml⁻¹ with isopropyl 1-thio-β-d-galactopyranoside, and the incubation continued for a further 5 h when the cells were harvested by centrifugation at 10,000 × g for 10 min. Harvested cells were stored at −18 °C until needed, when they were thawed on ice, and ~50 g of cells were sonicated in 500 ml of 0.1 M Tris-HCl, 0.3 M NaCl, pH 8.0 (buffer 1). The sonicated material was centrifuged at 10,000 × g for 40 min, and the decanted supernatant was loaded onto a 25-ml Probond column. The column was then washed with 500 ml of buffer 1 and subsequently eluted with a 1-liter 0.0–0.3 M imidazole gradient in buffer 1, collecting 10-ml fractions. GRP78-containing fractions were located by SDS-PAGE analysis, and appropriate fractions were pooled and dialyzed against 2 × 5-liter changes of 50 mM potassium phosphate, pH 7.2, 1 mM dithiothreitol (buffer 2). Using an AKTA explorer, the dialyzed pool of protein was then loaded in sixths onto a Mono Q HR 10/10 FPLC column. The column was then washed with 3 column volumes of buffer 2 and eluted with a 20-column volume gradient of 0.0–1.0 M NaCl in buffer 2, collecting 2-ml fractions at a flow rate of 2.5 ml min⁻¹. GRP78-containing fractions were identified by SDS-PAGE and showed that it eluted as two overlapping peaks. Samples from each peak were then analyzed by size exclusion chromatography using a Superdex 200 HR 10/30 column using a buffer consisting of 50 mM potassium phosphate, pH 7.2, 1 mM dithiothreitol, 150 mM NaCl (buffer 3). Samples from the first peak were found to elute as a single peak from the column at an estimated molecular mass of ~70 kDa, whereas the second peak showed two species eluting at estimated masses of 70 and >200 kDa. The fractions from the MonoQ column containing the first peak of GRP78 were pooled and pressure-concentrated to a volume of 25 ml. The concentrated pool was then loaded on to a Sephacryl S300 column of dimensions 115 × 2.5 cm that had been previously equilibrated with 50 mM potassium phosphate, pH 7.2, 0.75 M NaCl, 1 mM β-mercaptoethanol (buffer 4). GRP78-containing fractions were located by SDS-PAGE, pooled, and dialyzed against 3 × 5-liter changes of buffer 2. The dialyzed protein was then loaded onto a hydroxyapatite column of dimensions 6.5 × 5 cm, washed with 450 ml of buffer 2, and the column was eluted with a 50–400 mM potassium phosphate (pH 7.2, containing 1 mM dithiothreitol) gradient. GRP78-containing fractions were located by SDS-PAGE and pooled, with a typical preparation yielding ~125 mg of GRP78 at a purity greater than 95%. The affinity of the protein for Ca²⁺ (as measured by DSC) was used as a quality control assay for protein purification. GRP78 purified by this method was found to greatly reduce batch to batch variation (up to a 2-fold difference in the kₐ for Ca²⁺ binding) found when alternative purification protocols were used. The cDNA sequences encoding GRP78 encoding in plasmids pHis6-Bip (wild type GRP78) and pHis6-Bip:G565R were determined by direct sequence analysis and were identical to that published previously (24, 25).

**Circular Dichroism Spectroscopy**—The observed change in the tertiary structure of GRP78 in the presence of ATP or ADP was probed by near-UV CD spectroscopy. Near-UV CD spectra were recorded from an average of five cumulative scans at 20 °C from 320 to 250 nm using a Jasco J-810 spectropolarimeter. Samples of GRP78 in 50 mM HEPES, pH 7.2, 1 mM β-mercaptoethanol were used at concentrations between 8 and 12 μM. The change in the near-UV signal was monitored by the addition of ATP or ADP in the final concentration range 0–100 μM. The change in the near-UV signal was also monitored in the presence of 5 mM CaCl₂ by the addition of ATP in the final concentration range 0–80 μM. Each set of ATP or ADP titrations was carried out in triplicate, and each spectrum was base line-corrected by subtraction of a comparative blank. Similarly, ATP titrations were carried in triplicate in the presence of 5 mM CaCl₂. The 265 nm reading for each titration was used to calculate the kₐ for binding ATP or ADP in the absence of CaCl₂ and ATP in the presence of CaCl₂. The 265 nm readings for each sample were corrected by subtracting the 265 nm reading in the absence of ATP or ADP from each of the readings in the data set. The corrected 265 nm data were fitted to a single-site model, by nonlinear least-squares regression, using Scientist as described in Ref. 26. Control experiments were carried out, titrating GTP into GRP78 in the concentration range 0–80 μM and CaCl₂ into Grp78 in the concentration range 0–8 mM, and no change in near-UV CD signal at 265 nm was observed.

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry (ITC) is a nondestructive and noninvasive (label-free) technique that can be used to give a direct measure of the kₐ and additionally provides information on the enthalpy (∆H) and stoichiometry (n) of ligand binding. In these ITC experiments, a protein solution is placed in a reaction cell and heated at a constant rate, while the calorimeter is scanned in the temperature range of interest. The change in near-UV CD signal at 265 nm was observed.
Ligand Binding Properties of GRP78

vessel, and ligands are titrated in from a syringe while constantly stirring the mixture. Any heat absorbed (endothermic reaction) or released (exothermic reaction) when ligand binds to the protein is measured directly. Standard Gibbs free energy ($\Delta G^\circ$) and entropy ($\Delta S^\circ$) of ligand binding are subsequently calculated from the following equations.

$$\Delta G^\circ = -RT \ln K_b = +RT \ln K_d = \Delta H - T \Delta S^\circ$$  
(Eq. 1)

ITC experiments were performed at 25 °C using a high precision VP-ITC system (Microcal Inc.). GRP78 was dialyzed into 50 mM HEPES, pH 7.2, 1 mM $\beta$-mercaptoethanol. The concentrations of GRP78, nucleotides, and CaCl$_2$ used in the injector and cell are shown in Table 3. The heat evolved following each 10-μl injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction was obtained as the difference between the heat of reaction and the corresponding heat of dilution. Analyses of data were performed using Microcal Origin software.

**Differential Scanning Calorimetry—**DSC is a sensitive technique that measures the heat energy uptake (excess heat capacity) during thermally induced transitions in solution. In the case of proteins, analysis of the DSC thermogram can give detailed information about the mechanism of the unfolding transition, including the level of cooperativity, reversibility, and number of domains involved, together with the effects of bound ligands. DSC data can be used to give two independent estimates of the thermodynamic enthalpy (heat energy uptake) associated with the unfolding transition; the calorimetric enthalpy ($\Delta H_{cal}$) derives from the integrated area under a thermogram peak and is an absolute measure that depends on the concentration and integrity of the protein sample, whereas the van’t Hoff enthalpy ($\Delta H_{vth}$) depends only on the shape of the transition (29, 30). In ideal circumstances, these quantities are the same, and the calorimetric/van’t Hoff enthalpy ratio is close to unity. Deviations from this behavior indicate the presence of impure or misfolded protein, irreversibility, and/or lack of cooperativity in the unfolding transition. Binding of ligands to the native protein will naturally stabilize the folded form, and this will be manifest in an observed increase in the midpoint temperature ($T_m$) for the protein (or domain) involved in the transition. For a ligand that binds to the native (folded) state of a protein, the increase in $T_m$ can be related to the dissociation constant ($K_d$) and concentration of ligand, [L], as shown in the following equation (29, 30).

$$\delta T_m / T_m = \left( RT_m / \Delta H_{vth} \right) \ln(1 + [L]/K_d)$$  
(Eq. 2)

in which $\delta T_m = T_m - T_m^{0}$ is the change in unfolding transition temperature, $R$ is the gas constant, and $\Delta H_{vth}$ is the unfolding enthalpy of the protein domain in the absence of ligand.

DSC measurements on Grp78 were made using a Microcal VP-DSC instrument at a scan rate of 90 °C/1 h and a protein concentration in the range 13–23 μM. Protein was dialyzed into 50 mM HEPES, pH 7.2, 1 mM $\beta$-mercaptoethanol, and the dialysis buffer was retained to dissolve ligands and for base line controls. Each sample was heated from 25 to 32 °C and cooled to 25 °C and then heated from 25 to 80 °C. Deconvolution analysis was performed using the non-two-state model provided with Microcal Origin software.

**Protein Concentrations—**Protein concentrations were measured spectrophotometrically as described previously (27), using the theoretical molar absorption coefficient calculated by the Vector Nti suite of programs and will therefore have an error of up to 5%.

**SDS-PAGE Analysis of Proteins—**SDS-PAGE, using a 10% acrylamide separating gel, was carried out as previously described (28).

**RESULTS**

**Purification and Characterization of the Wild Type and G565R Forms of GRP78—**Overproduction of the wild type untagged GRP78 protein has previously been reported to be toxic to *E. coli*, whereas overproduction of a His$_{6}$-tagged form is less toxic and overproduction of a His$_{6}$-tagged G565R mutant form is nontoxic (22). The functional integrity of the G565R GRP78 was previously assessed by ATPase activity in the presence of the peptide LSVKFLT and the ability to interact with the J-domain of the DnaJ-like accessory protein MY1 and found to have near wild type values (22). Since we wished to carry out a full thermodynamic analysis of Ca$^{2+}$ and nucleotide binding to GRP78, we initiated studies with the G565R mutant form, since this had been reported to be overproduced at a higher level. In order to be confident that the properties of the mutant form were functionally equivalent to the wild type, we planned to purify a small quantity of the wild type protein for a limited comparative analysis. However, under our induction conditions, the wild type His$_{6}$-tagged GRP78 was not toxic in *E. coli*, and we were able to purify it in the same quantities as the G565R form. Therefore, we carried out a full comparison of wild type and G565R GRP78 to assess whether the G565R mutation had any significant effects on the binding of Ca$^{2+}$ and nucleotides. Wild type and G565R GRP78 were purified using the protocol described under “Experimental Procedures.” Fig. 1 shows examples of both forms of the protein analyzed by SDS-PAGE. These proteins were compared by far-UV CD analysis and found to be indistinguishable (data not shown). Table 1 shows that independent preparations of both forms of GRP78 purified by this method showed good agreement in terms of the positions of the peaks of the thermal transitions revealed by DSC.

**Differential Scanning Calorimetry Reveals that GRP78 Unfolds as Two Independent Domains—**The purified GRP78 proteins underwent two thermal transitions, and a typical thermogram of unliganded wild type GRP78 is shown in Fig. 2A. Fig. 2B shows an unfolding of the first domain, and a second heating shown in Fig. 2C shows that the unfolding of this domain is partially reversible. This sample was reheated for a third time (Fig. 2D) and again shows the partial reversibility of refolding of the first domain and independent unfolding of the second domain. The sequential scans in Fig. 2, B–D, show that for wild type GRP78 protein, the unfolding of the two domains is independent. However, when GRP78 is heated from 25 to 80 °C (Fig. 2E) and then reheated (Fig. 2F), the unfolding of both domains shows little reversibility. The same unfolding and refolding pattern was observed with G565R mutant GRP78 (data not shown). Table 1 shows that for the
TABLE 1

Thermodynamic parameters for the unfolding of wild type and G565R GRP78 as determined by DSC in the presence of ligands

The unfolding of three independent preparations of wild type and G565R GRP78 was measured in 50 mM HEPES, pH 7.2, 1 mM β-mercaptoethanol. Shown are the values for $T_m$, the midpoint of the thermal transition; $K_D$, the equilibrium dissociation constant; $\Delta H_{cal}$, the calorimetric enthalpy; and $\Delta H_{vH}$, the van’t Hoff enthalpy. The values shown are the average (rounded to one decimal place) of three determinations each from three independent preparations of the two forms of GRP78. The S.D. values are shown in parentheses.

| Sample | $T_m$ (°C) | $\Delta H_{cal}$ (kcal mol$^{-1}$) | $\Delta H_{vH}$ (kcal mol$^{-1}$) | $T_m$ (°C) | $\Delta H_{cal}$ (kcal mol$^{-1}$) | $\Delta H_{vH}$ (kcal mol$^{-1}$) |
|--------|------------|-----------------------------------|-----------------------------------|------------|-----------------------------------|-----------------------------------|
| Wild type GRP78 | 46.2 (0.5) | 151 (10) | 153 (2) | 67.0 (0.1) | 125 (5) | 167 (5) |
| G565R GRP78 | 46.6 (0.2) | 154 (2) | 154 (2) | 66.0 (0.3) | 128 (9) | 138 (1) |

GRP78 binds Ca$^{2+}$, Mg$^{2+}$, ATP, and ADP Cooperatively—In order to investigate and quantify the ligand binding properties of GRP78, we carried out a microcalorimetry-based analysis in the presence of Ca$^{2+}$, ATP, and ADP and mixtures thereof. We chose DSC for the initial analysis, because it allows the detection of very weak ligand binding and has the added advantage that it requires substantially less protein than ITC. For these experiments, we initially analyzed thermograms encompassing both thermal transitions and found that when the ligands were used individually only the $T_m$ of the first transition was affected (see Table 2). By comparison with the structure of the GRP78 homologue Hsp70, we are able to infer that the first transition corresponds to the unfolding of the N-terminal domain in GRP78, since the N-terminal domain of Hsp70 has been shown to bind ATP (29). When Ca$^{2+}$ is bound to GRP78 the upward shift in $T_m$ of the first peak makes accurate fitting of the base line to the second peak difficult. Therefore, the calculations of the calorimetric to van’t Hoff for the second transition in the liganded forms are not shown. The problem is exacerbated when ATP, ADP, or a combination of nucleotide and Ca$^{2+}$ is added to GRP78, since the upward shift in $T_m$ is so great that it is not possible to accurately fit base lines to either transition. In these cases, the $T_m$ values of the first transition are shown with no further analysis.

The increase in $T_m$ of the first thermal transition but not the second in the presence of Ca$^{2+}$, ATP, or ADP strongly supports the view that the ligands are binding to the same domain within GRP78. The analysis was then repeated using ATP or ADP in combination with Ca$^{2+}$, but in this case the temperature range used in the thermograms only encompassed the first thermal transition. Table 2 summarizes the thermodynamic parameters associated with the unfolding of GRP78 in the presence of the various combinations of these ligands and shows that Ca$^{2+}$ binds to the wild type and G565R forms with an average $K_D$ of ~0.6 mM (30, 31). Inspection of Table 2 shows that for both forms of the protein, the increase in $T_m$ in the presence of divalent cation and ATP or ADP is greater than the sum of the increases when the same concentrations of ligand are used separately. This is consistent with ITC data (see below) indicating that divalent cations and ATP or ADP bind to GRP78 in a cooperative manner so that the $T_m$ shifts are not simply additive.

An important observation derived from these data is that Ca$^{2+}$ has a differential cooperative effect on the binding of ADP and ATP to GRP78. Table 2 shows that the upward shift in the $T_m$ of GRP78 in the presence of Ca$^{2+}$, and ADP is much greater than Ca$^{2+}$ and ATP ($\Delta T_m$ of 11.9 versus 8.4 for wild type protein). This observation implies that the $K_D$ values for the Ca$^{2+}$/ADP combination are cooperatively enhanced to a greater degree than that of the Ca$^{2+}$/ATP combination.

In order to determine whether divalent metal cations other than Ca$^{2+}$ have a similar effect on ADP/ATP binding to GRP78, we carried out a DSC analysis of nucleotide binding in the presence of Mg$^{2+}$. Table 2 shows that Mg$^{2+}$ binds to GRP78 with an approximate $K_D$ of 0.4 mM and that, like Ca$^{2+}$, this binding is cooperative with ADP and ATP. However, the increase in the $T_m$ of the first transition of GRP78 is increased to approximately the same extent in mixtures of Mg$^{2+}$/ADP and Mg$^{2+}$/ATP ($\Delta T_m$ of 9.0 versus 8.6 for wild type protein), implying that the $K_D$ values for the Mg$^{2+}$/ADP combination are not cooperatively enhanced to a greater degree than that of the Ca$^{2+}$/ATP combination.

GRP78 binds Ca$^{2+}$ with 1:1 Stoichiometry—We used ITC to measure the stoichiometry of binding for the important cell signaling cation Ca$^{2+}$, in the presence and absence of ADP and ATP. We first looked at the binding of Ca$^{2+}$ in the absence of ADP or ATP; however, the binding was undetectable by this method at the Ca$^{2+}$ concentration used (3 mM in the injection syringe, translating to around 0.3 mM in the final reaction mixture) and is consistent with the weak...
binding revealed by DSC. The experiments were repeated in the presence of ADP or ATP at 1 mM. Fig. 3 shows typical thermograms, and Table 3 summarizes the associated thermodynamic parameters. These data show that for each protein, the results are essentially identical and that in each case a single-site (1:1 stoichiometry) binding model adequately describes the data.

**TABLE 2**

Thermodynamic parameters for the binding of Ca\(^{2+}\), Mg\(^{2+}\), ATP, and ADP to G565R and wild type GRP78 as determined by DSC

The binding of divalent metal cations, ATP, and ADP to GRP78 was measured in 50 mM HEPES, pH 7.2, 1 mM β-mercaptoethanol, using GRP78 at 23 μM, divalent metal cations at 1 mM, and ATP/ADP at 1 mM. Shown are the values for \(T_m\), the midpoint of the thermal transition; \(K_d\), the equilibrium dissociation constant; \(\Delta H_{cal}\), the calorimetric enthalpy; and \(\Delta H_{VH}\), the van’t Hoff enthalpy. The values shown are the average (rounded to one decimal place) of three independent determinations, and the S.D. values are shown in parentheses. NA, not applicable.

| Sample                  | \(T_m\) °C | \(\Delta T_m\) °C | \(\Delta H_{cal}\) kcal mol\(^{-1}\) | \(\Delta H_{VH}\) kcal mol\(^{-1}\) | \(K_d\) mM |
|-------------------------|------------|-------------------|-------------------------------------|---------------------------------|-----------|
| **Wild type GRP78**     |            |                   |                                     |                                 |           |
| GRP78 (23 μM) unliganded| 46.7 (0.1) | 1.2               | 153 (19)                            | 146 (13)                        | 0.7 (0.2) |
| GRP78 (23 μM) + 1 mM CaCl\(_2\) | 47.9 (0.2) | 1.2               | 153 (19)                            | 146 (13)                        | NA        |
| GRP78 (23 μM) + 1 mM ATP | 49.6 (0.4) | 2.9               | 153 (19)                            | 146 (13)                        | NA        |
| GRP78 (23 μM) + 1 mM ADP | 53.1 (0.1) | 6.4               | 153 (19)                            | 146 (13)                        | NA        |
| GRP78 (23 μM) + 1 mM CaCl\(_2\) + 1 mM ATP | 55.1 (0.1) | 8.4               | 153 (19)                            | 146 (13)                        | NA        |
| GRP78 (23 μM) + 1 mM ADP | 58.6 (0.1) | 11.9              | 153 (19)                            | 146 (13)                        | NA        |
| GRP78 (23 μM) + 1 mM MgCl\(_2\) | 48.4 (0.1) | 1.7               | 151 (14)                            | 161 (9)                         | 0.4 (0.1) |
| GRP78 (23 μM) + 1 mM MgCl\(_2\) + 1 mM ATP | 55.3 (0.2) | 8.6               | 138 (55)                            | 194 (2)                         | NA        |
| GRP78 (23 μM) + 1 mM MgCl\(_2\) + 1 mM ADP | 55.7 (0.2) | 9.0               | 210 (9)                             | 190 (3)                         | NA        |

| **G565R GRP78**         |            |                   |                                     |                                 |           |
| GRP78 (23 μM) unliganded| 46.6 (0.1) | 1.5               | 159 (9)                             | 159 (3)                         | 0.5 (0.1) |
| GRP78 (23 μM) + 1 mM CaCl\(_2\) | 48.0 (0.2) | 1.5               | 159 (9)                             | 159 (3)                         | NA        |
| GRP78 (23 μM) + 1 mM ATP | 50.2 (0.3) | 3.7               | 159 (9)                             | 159 (3)                         | NA        |
| GRP78 (23 μM) + 1 mM ADP | 52.6 (0.2) | 6.1               | 159 (9)                             | 159 (3)                         | NA        |
| GRP78 (23 μM) + 1 mM CaCl\(_2\) + 1 mM ATP | 55.2 (0.1) | 8.7               | 159 (9)                             | 159 (3)                         | NA        |
| GRP78 (23 μM) + 1 mM ADP | 58.9 (0.1) | 12.4              | 159 (9)                             | 159 (3)                         | NA        |
| GRP78 (23 μM) + 1 mM MgCl\(_2\) | 48.3 (0.1) | 1.7               | 166 (7)                             | 161 (2)                         | 0.4 (0.1) |
| GRP78 (23 μM) + 1 mM MgCl\(_2\) + 1 mM ATP | 55.9 (0.1) | 9.3               | 223 (4)                             | 163 (3)                         | NA        |
| GRP78 (23 μM) + 1 mM MgCl\(_2\) + 1 mM ADP | 55.6 (0.1) | 9.0               | 277 (5)                             | 183 (2)                         | NA        |

\(a\) Estimated using the approximate expression symbol \(\Delta T_m/T_m = (RT_m/\Delta H)\ln(1 + (1/K_d))\) (30, 31).
The Binding of Ca\(^{2+}\) to GRP78 Is Differentially Enhanced by ADP and ATP—The data in Table 3 show that equivalent concentrations of ADP and ATP (1 mM) have a differential effect on the cooperative binding of Ca\(^{2+}\), with ADP causing an ~22-fold (wild-type protein) or 9-fold (G565R protein) greater effect on the KD for Ca\(^{2+}\) binding relative to ATP. These large decreases in the KD values for Ca\(^{2+}\) binding in the presence of ADP or ATP are consistent with the DSC data that show the binding of Ca\(^{2+}\) and ADP/ATP is cooperative (see Table 2). Caution must be exercised in the interpretation of the absolute values of the KD values given for Ca\(^{2+}\) binding in the presence of 1 mM ATP or ADP, since these are apparent values (KD(app)). This is because the KD values for all of the interactions of GRP78 (protein/Ca\(^{2+}\), protein/nucleotide, and protein/Ca\(^{2+}\)/nucleotide) are linked to one another and to the concentrations of ATP, ADP, and Ca\(^{2+}\) used in the experiments described. However, the concentrations of ATP and ADP used (1 mM) are large compared with the measured KD for nucleotide binding to GRP78 and the calculated KD for the binding of nucleotide to Ca\(^{2+}\)-saturated GRP78. Therefore, the KD(app) for Ca\(^{2+}\) binding to GRP78 in the presence of 1 mM nucleotide will be reasonably close to the value that would be obtained with pure GRP78-nucleotide complex. See Appendix 1 for further details.

The Binding of ADP and ATP to GRP78 Is Differentially Enhanced by Ca\(^{2+}\)—We investigated by ITC the binding of ATP and ADP to GRP78 (see Table 3) and found that ADP binds to wild-type GRP78 with a 1:1 stoichiometry and a KD of 25 M and to G565R GRP78 with a 1:1 stoichiometry and a KD of 1.4 M (see Fig. 4A). When the ITC experiments with ADP were repeated in the presence of 1 mM Ca\(^{2+}\), the binding of ADP to GRP78 was enhanced to such a degree that it was beyond the limits of sensitivity for the ITC technique (data not shown).

The binding of ATP is complex for the wild-type and G565R proteins (see Fig. 4B for a typical example using wild-type protein), and this may result from a low level ATPase activity associated with GRP78. In order to try and circumvent this problem, we attempted to measure the KD for ATP binding to GRP78 by using CD measurements. The rationale behind these experiments is that the presence of ATP has been reported to disrupt the interaction between GRP78 and caspase-12 in vitro (19). It was possible that ATP may cause GRP78 to undergo some conformational changes that affect caspase-12 binding and that this change could be monitored in vitro by CD measurements in the presence of increasing concentrations of ATP or ADP.

In order to test this possibility, we looked for evidence of changes in the tertiary structure of GRP78 in the presence of ATP or ADP by near-UV CD spectroscopy. GRP78 has two tryptophan residues, one in the N-terminal domain and one in the C-terminal domain. The C-terminal tryptophan is in an irrelevant position, but the N-terminal residue had the potential to be useful as a probe for conformational changes if it was not in a surface-exposed region. Preliminary experiments showed that when ATP or ADP was added to GRP78, the greatest changes in signal were generated in the region of 255–270 nm, a region of the CD spectrum dominated by phenylalanine (32). The relatively weak signal in the CD spectrum, 275–305 nm, characteristic of tryptophan (32), implies that the N-terminal tryptophan is either in a buried conformation that does not give a signal (33) or is in a surface-exposed region. The increasing molecular CD values recorded in the CD spectrum 255–270 nm are unlikely to be due to GRP78-induced changes in the ADP/ATP, because the molecular CD values for ADP/ATP in buffer alone are increasingly negative with increasing concentration.

Fig. 5 shows the effect of increasing concentrations of ATP on the signal of G565R GRP78 measured in the region 250–320 nm, and the increasing signal at 265 nm was used as a measure of the change in the environment of phenylalanine residues. This experiment was carried out three times, and when a linear least squares regression program (26) using Scientist software was used to plot the measurements at 265 nm as a function of ATP concentration for each data set, an average KD value of 14.5 ± 4 M for the binding of ATP to G565R GRP78 was obtained.

The experiments were repeated with the wild-type protein giving a KD of 13.8 ± 2 M. The results of a typical experiment with G565R GRP78 are shown in Figs. 5 and 6. Similar CD spectra (data not shown) were obtained when the experiment was repeated three times.

### Table 3

| Ligand tested | [Ligand] in syringe | [GRP78] in cell | n | KD(app) | Average KD(app) | ΔHobs | ΔS | c |
|---------------|---------------------|----------------|---|---------|----------------|-------|----|---|
| Ca\(^{2+}\) binding to wild type | 1 mM CaCl\(_2\) + 1 mM ATP | 36 μM + 1 mM ATP | 1.0 (0.1) | 14.4 | 1.7 (0.1) | 27.9 | 2.5 |
| GRP78 in the presence of ATP | 1 mM CaCl\(_2\) + 1 mM ATP | 36 μM + 1 mM ATP | 1.1 (0.2) | 20.6 | 1.6 (0.1) | 26.9 | 1.5 |
| Ca\(^{2+}\) binding to wild type | 1 mM CaCl\(_2\) + 1 mM ATP | 36 μM + 1 mM ATP | 1.0 (0.1) | 18.7 | 1.8 (0.1) | 27.5 | 1.9 |
| GRP78 in the presence of ATP | 0.5 mM CaCl\(_2\) + 1 mM ATP | 36 μM + 1 mM ATP | 0.7 (0.1) | 0.9 | 0.8 ± 0.1 | 5.5 (0.1) | 9.8 | 54 |
| ADP binding to wild type | 1 mM ADP | 27 μM | 0.7 (0.1) | 2.7 | 2.5 ± 0.4 | 9.5 (0.1) | 8.8 | 9.5 |
| GRP78 | 1 mM ADP | 27 μM | 0.8 (0.1) | 2.0 | 2.5 ± 0.4 | 9.5 (0.1) | 8.8 | 9.5 |
| ADP binding to G565R | 1 mM ADP | 27 μM | 0.8 (0.1) | 2.8 | 2.5 ± 0.4 | 9.5 (0.1) | 8.8 | 9.5 |
| GRP78 in the presence of ATP | 0.75 mM CaCl\(_2\) + 1 mM ADP | 34 μM + 1 mM ATP | 1.0 (0.1) | 18.0 | 20.1 ± 2.8 | 4.0 (0.2) | 35.1 | 1.9 |
| Ca\(^{2+}\) binding to G565R | 1 mM CaCl\(_2\) + 1 mM ATP | 34 μM + 1 mM ATP | 1.0 (0.1) | 2.3 | 4.2 (0.5) | 35.6 | 1.5 |
| GRP78 in the presence of ATP | 0.65 mM CaCl\(_2\) + 1 mM ADP | 34 μM + 1 mM ATP | 0.9 (0.1) | 2.4 | 2.2 ± 0.3 | 2.8 (0.01) | 16.8 | 15.3 |
| ADP binding to G565R | 1 mM ADP | 40 μM | 1.0 (0.1) | 1.7 | 1.4 ± 0.3 | 10.0 (0.1) | 6.9 | 34.5 |
| GRP78 | 0.8 mM ADP | 40 μM | 0.9 (0.1) | 1.2 | 1.4 ± 0.3 | 10.0 (0.1) | 6.9 | 34.5 |
| 0.5 mM ADP | 40 μM | 1.0 (0.1) | 1.2 | 1.4 ± 0.3 | 10.0 (0.1) | 6.9 | 34.5 |

The binding of Ca\(^{2+}\) to G565R and wild type GRP78 in the presence of 1 mM ATP or ADP was measured in 50 mM HEPES, pH 7.2, 1 mM β-mercaptoethanol by ITC. Additionally, the binding of ATP was determined at the concentration indicated. Shown are the values for the stoichiometry of binding; KD(app), the apparent equilibrium dissociation constant; ΔHobs, the observed enthalpy; and ΔS, the standard entropy change for single site binding. The c values fall within the range of 1–1000 that allows the isotherms to be accurately deconvoluted with reasonable confidence to derive K values (46). The average KD(app) values are shown ± S.D.
times in the presence of 5 mM Ca\(^{2+}\) for both the G565R and wild type GRP78. For the G565R GRP78, an average \(K_D\) value of 1.0 ± 0.2 mM, ~14-fold lower than ATP alone, was determined, and for the wild type protein, the \(K_D\) was approximately 1.2 ± 0.3 mM, around 11-fold lower than for ATP alone.

These values demonstrate that the binding of ATP and Ca\(^{2+}\) is cooperative, and the behaviors of the wild type and G565R GRP78 are essentially identical. We then determined the \(K_D\) for the binding of ADP to wild type and G565R GRP78 in the absence of Ca\(^{2+}\) by this method and obtained an average value of 2.8 ± 0.5 mM and 3 ± 0.6 mM, respectively, which compares favorably with the values (2.5 ± 0.4 mM for the wild type and 1.4 ± 0.3 mM for the G565R form) determined by ITC. This concurrence in the \(K_D\) values for ADP binding to GRP78 by the two methods gives confidence that the \(K_D\) value determined by CD analysis for ATP binding to GRP78 is likely to be reasonably accurate. When these experiments were repeated with the same concentrations of Ca\(^{2+}\) and ADP, we reproducibly observed precipitation of the GRP78 and consequently were unable to analyze the data. However, calculations based on the data for wild type GRP78 we report here predict that the \(K_D\) for ADP in the presence of Ca\(^{2+}\) will be lowered around 930-fold to 3 nM. Similar calculations for ATP in the presence of Ca\(^{2+}\) predict a \(K_D\) of 0.4 ± 0.3 M, which is close to the value of 1.2 µM we measured by CD. For details of these calculations see Appendix 1.

No change in the near-UV CD signal at 265 nm was observed when calcium alone or a control of GTP was added to GRP78. These observations are consistent with the ITC and DSC data that indicate that Ca\(^{2+}\) and ATP/ADP bind to GRP78 cooperatively.

**Ca\(^{2+}\) and Mg\(^{2+}\) Bind to the Same or Overlapping Sites within the N-terminal Domain of GRP78**—The DSC data show that the binding site for Ca\(^{2+}\) and Mg\(^{2+}\) is located within the N-terminal domain of GRP78; however, they give no indication as to whether the sites are identical, separate, or overlapping. In order to shed some light on these possibilities, we have used ITC to look at the interaction between Ca\(^{2+}\) and GRP78 in the presence of 0.5 mM Mg\(^{2+}\) and 1 mM ADP. When a mixture of 0.5 mM Ca\(^{2+}\), 0.5 mM Mg\(^{2+}\), and 1 mM ADP in the injector is titrated into a mixture of 0.5 mM Mg\(^{2+}\), 1 mM ADP, and 27 µM GRP78, no significant heat exchange over background is
observed (see Fig. 7). These data are consistent with Ca\(^{2+}\) and Mg\(^{2+}\) both competing for a common or overlapping region within the nucleotide binding site.

**DISCUSSION**

The literature regarding a possible role for GRP78 as a dynamic Ca\(^{2+}\) storage protein is confused. Concentrations of Ca\(^{2+}\) in the ER have been quoted to reach up to 10 mM, and the two basic properties expected for a dynamic Ca\(^{2+}\) storage function are high capacity, corresponding to 25–50 mol/mol, and low affinity, with a \(K_D\) in the millimolar range (11). We have addressed the involvement of GRP78 as a Ca\(^{2+}\) storage protein by using *in vitro* microcalorimetric methods at calcium concentrations that fall within the range found in the ER lumen. The advantages of ITC and DSC in this context are that, in addition to giving absolute thermodynamic data for the biomolecular interactions involved here, they also give direct information about the protein domains affected by ligand binding (DSC) and the absolute stoichiometries of ligand binding (ITC), especially relevant in this case to the calcium binding function.

Using these techniques, our results show for the first time that GRP78 has a relatively high \(K_D\) value (0.7 mM) for the binding of Ca\(^{2+}\) in the absence of ADP or ATP, and in the presence of saturating concentrations of these nucleotides the value is \(\approx 18 \mu M\) (around 40-fold lower) for ATP and 0.8 \(\mu M\) (around 880-fold lower) for ADP and with a 1:1 stoichiometry. These properties are not those of a classical dynamic Ca\(^{2+}\) storage protein. Nonetheless, overexpression in HeLa cells of hamster GRP78 to around 200\% of that in controls has been reported to induce appreciable increases of the ER Ca\(^{2+}\) storage capacity (5). Other experiments, in which tunicamycin was used to induce increased transcription of the host grp78 gene in the presence of \(^{45}\text{Ca}^{2+}\), showed that increased production of the endogenous GRP78 was paralleled by marked increases in \(^{45}\text{Ca}^{2+}\) release from the cells (5). These experiments have been interpreted to mean that under the conditions reported (5) GRP78 can play a role in the storage of the rapidly exchanging pool of Ca\(^{2+}\). However, it remains unclear if this potential property is significant under typical physiological conditions and GRP78 concentrations.

We have shown that GRP78 unfolds in two independent thermal transitions, a result most simply interpreted as meaning GRP78 has two domains, consistent with the structures of other members of the Hsp70 family (8), and the data in Fig. 2 imply that the N-terminal ATP-binding domain is the first to unfold. Ca\(^{2+}\), ATP, and ADP all bind to this domain in a cooperative manner, with the binding of ATP/ADP causing a conformational change. Our data show that the presence of Ca\(^{2+}\) lowers the \(K_D\) for the binding of ATP to GRP78 by around 11-fold, and calculations based on this measured value (see Appendix 1) predict that saturating concentrations of Ca\(^{2+}\) should lower the \(K_D\) for ADP by around a factor of 930 to the low nanomolar range. Similarly, DSC analysis shows that the cation Mg\(^{2+}\) binds cooperatively with ATP and ADP. However, Mg\(^{2+}\) causes approximately the same upward shift in the \(T_m\) in the presence of ATP (\(\Delta T_m\) 8.6 °C) or ADP (\(\Delta T_m\) 9.0 °C) in contrast to Ca\(^{2+}\), which causes a \(\Delta T_m\) of 8.4 °C with ATP and a \(\Delta T_m\) of 11.9 °C with ADP. These observations imply that, in contrast to Ca\(^{2+}\), Mg\(^{2+}\) enhances the affinity of ATP and ADP for GRP78 to approximately the same extent. Based on equilibrium dialysis experiments in the presence of 2 mM Mg\(^{2+}\), it has been reported previously that GRP78 from hamster binds ATP and ADP to approximately the same extent (34).

GRP78 is mainly located in the ER lumen, where Ca\(^{2+}\) concentrations are at their highest; therefore, these observations provide a potential molecular mechanism for the requirement of a specific nucleotide exchange factor to remove bound ADP from GRP78. The protein designated BAP (a mammalian BiP-associated protein) is present in the lumen of the endoplasmic reticulum, and it serves as a nucleotide exchange factor for GRP78, removing bound ADP from the protein (35). The chaperone activity of GRP78 is controlled by ATP hydrolysis in what is termed the ATPase cycle (36). In brief, ATP-bound GRP78 is proposed to be in an “open” form that can be recruited to bind unfolded regions of target proteins. Stimulation of GRP78 ATPase activity converts it to a “closed” ADP-bound form that locks it on to its target protein. BAP is proposed to remove ADP from GRP78, converting it back to its “open” form, leading to release from its protein substrate (36). GRP78 carries out its chaperonin function within the ER lumen and so is exposed to concentrations of calcium in the millimolar range (37). The effective \(K_D\) of GRP78 will be in the low nanomolar range for ADP compared with low \(\mu M\) for ATP, implying that in the presence of a mixture of ATP/ADP, a significant proportion of the GRP78 population...
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will be in the inactive “closed” form and not readily available for binding to new protein substrates. Under these conditions, the role of BAP will be crucial to remove the ADP from GRP78, returning it to its open form that is capable of fulfiling its chaperone function. The binding of ATP and ADP to bovine 70-kDa heat shock cognate protein (Hsc70) under a number of different conditions has been reported (38–41), and values in the approximate range 10^{-7} to 10^{-8} M have been described (41). Our data for the wild type and G565R GRP78 are close to the K_d values reported for the binding of ATP (13.8±14.5 versus 9.5 μM) and ADP (2.5±1.4 versus 1.6 μM) to bovine Hsc70 in the absence of Mg^{2+} (38), but other enzymatic studies have reported that ATP binds more tightly than ADP to Hsc70 (39–41). The G565R form of GRP78 has previously been shown to retain its ATPase catalytic activity and to be fully functional (22).

It has been reported that the presence of ATP causes GRP78 to dissociate from caspase-12 (19). Our results demonstrate directly for the first time that the binding of ATP/ADP does induce conformational changes in GRP78, consistent with a model in which an ATP-induced conformational change may be responsible for the dissociation of GRP78 from caspase-12. It is tempting to speculate that the differential cooperative binding of ADP and ATP to GRP78 in the presence of Ca^{2+} has some regulatory role in influencing the interaction between GRP78 and the various components of the apoptosis signal transduction pathway. In support of this speculation, we have shown that Mg^{2+} enhances cooperative binding of ADP and ATP to GRP78 to approximately the same extent, implying that the differential effect seen with the cell signaling cation Ca^{2+} is specific and may have a biological function.

It is not clear at this stage whether Ca^{2+} binding involves a separate cation binding site on the protein or whether the divalent cation is more intimately involved as a necessary counterion to the di-/triphosphate group in the nucleotide binding site. However the data in Fig. 7 show that in the presence of ADP, premerging GRP78 with Mg^{2+} markedly reduces the binding of added Ca^{2+} in ITC experiments. These observations are consistent with Ca^{2+} and Mg^{2+} both competing for a common or overlapping region within the nucleotide binding site of GRP78. In this regard, it is interesting to note that the GRP78 homologue Hsp70, when crystallized, can bind two calcium ions within the ATPase domain (29). One site binds within the catalytic pocket, bridging ADP and inorganic phosphate, and the second is coordinated on the protein surface by Glu^{231}, Asp^{232}, and the carbonyl of His^{227} (29). The crystallization conditions in these experiments included CaCl_2 at the nonphysiological concentration of 20 mM, and there are no solution binding studies to provide data on the K_d for calcium binding to either site (29). Our experiments use concentrations of Ca^{2+} over an order of magnitude lower, falling within the range reported for the ER lumen (11), and at these more physiological Ca^{2+} concentrations, we find a single major binding site in GRP78 that can be competed out by the presence of Mg^{2+}. These observations are consistent with this major Ca^{2+} binding site in GRP78 corresponding to the catalytic pocket Mg^{2+} binding site in Hsp70. The amino acid residues that coordinate the second Ca^{2+} binding site in Hsp70 are conserved in GRP78, Hsc70, and adenylate kinase (42), implying that GRP78 has the potential for a second Ca^{2+} binding site. If this is the case, then the second putative Ca^{2+} binding site is likely to have a high K_d, since the ITC binding isotherms for Ca^{2+} binding in the presence of ATP are adequately described by a single site binding model (see Fig. 3). However, a detailed inspection of Fig. 3 shows that although the binding isotherms is consistent with a single site binding model, the titration of Ca^{2+} onto GRP78 in the presence of ATP does not return fully to zero. This is consistent with the presence of a second very weak, low substituted Ca^{2+} binding site. Interestingly, the titration of Ca^{2+} in the presence of ADP does return to zero, consistent with a single site binding model. Given this mixture of binding and crystallographic data, the presence of a second very low affinity (and, at physiological Ca^{2+} concentrations, low substituted) Ca^{2+} binding site on GRP78 cannot be ruled out. The potential biological relevance of such a putative low affinity binding site at physiological Ca^{2+} concentrations is unclear. A less likely but formal possibility is that the second Ca^{2+} binding site in Hsp70 has an extremely low K_d for Ca^{2+} binding that causes the protein to fill this site by scavenging Ca^{2+} during the purification procedure. Hsc70 is a homologue of Hsp70 and GRP78 that contains the Glu, Asp, and His residues reported to form the second Ca^{2+} Hsp70 binding site. The structure of Hsp70 reported (43) was determined from crystals formed in the absence of added Ca^{2+} and does not show the presence of Ca^{2+}. These observations therefore provide no support for the view that the conserved Glu, Asp, and His residues are an essential part of a high affinity Ca^{2+} binding site.

It is worth noting that, although Ca^{2+} binding is significantly enhanced in the presence of nucleotides, the underlying thermodynamics show some differences. In particular, binding of Ca^{2+} to GRP78 is endothermic in the presence of ATP but exothermic with ADP (Table 3 and Fig. 3). We have no obvious explanation for this, and there are likely to be a number of competing factors involved. For example, it may be that the unfavorable (endothermic) contribution to cation binding free energy in the presence of ATP (partially compensated by the more positive entropy contribution) might reflect the larger desolvation effects of counterion involvement with the triphosphate moiety. These solvation effects might be less significant in the case of ADP, consistent with its higher binding affinity. Ligand-induced conformational changes in the protein will also contribute to the overall thermodynamics, although such effects are usually compensatory and difficult to predict or rationalize (44).

Finally, in view of the use of mutant protein in previous studies (22), it is reassuring to report that the thermodynamic properties of the wild type and G565R forms of GRP78 are essentially identical.

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