Characterization of the Nucleotide Binding Properties and ATPase Activity of Recombinant Hamster BiP Purified from Bacteria*

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Jueyang Wei‡ and Linda M. Hendershot§

From the ‡Department of Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105 and the §Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38163

HSP70 family proteins bind ATP and hydrolyze it, but the precise role of these activities in their in vivo chaperoning function has not been determined. In this report, we characterized wild-type hamster BiP isolated from bacteria in terms of its ATP binding and ATPase activities. Recombinant BiP behaved essentially the same as endogenous BiP in terms of oligomeric status, protease digestion patterns, and ATPase properties. By engineering a Factor Xa cleavable site following the His tag which was used for affinity purification, we demonstrated that the six histidines had no effect on either the structural or ATPase properties of recombinant BiP. We also found that bacteria-synthesized BiP had a tightly bound ADP that was resistant to dialysis. Removal of the bound nucleotide allowed us to directly measure the binding affinity of ATP and ADP to BiP (Kd of 0.2 μM for ATP and 0.29 μM for ADP) by equilibrium dialysis. Careful characterization of wild-type BiP will allow us to use this system to characterize BiP ATP binding site mutants that can be used to probe the role of ATP binding and ATPase activity in BiP functions.

The heat shock protein 70 (HSP70) family is comprised of a highly conserved class of molecular chaperones. Members of this family exist in all species from Escherichia coli to human and in all the organelles of eukaryotic cells (1, 2). Most of these proteins are constitutively expressed and are further induced under conditions of stress like heat shock or other physiological insults to the cells (1). Immunoglobulin heavy chain binding protein (BiP) is the eukaryotic endoplasmic reticulum (ER) member of the HSP70 family, and is one of the major resident ER proteins. BiP is proposed to play a role in protein folding, subunit assembly, and subsequent transport of proteins from the ER (2). BiP associates transiently with a variety of nascent secretory proteins (3–7) and more stably with malfolded or unassembled proteins whose transport from the ER is blocked (2). BiP associates transiently with a variety of nascent secretory proteins (3–7) and more stably with malfolded or unassembled proteins whose transport from the ER (2). BiP is proposed to play a role in protein folding, subunit assembly, and subsequent transport of proteins from the ER (2). BiP associates transiently with a variety of nascent secretory proteins (3–7) and more stably with malfolded or unassembled proteins whose transport from the ER is blocked (2). BiP is proposed to play a role in protein folding, subunit assembly, and subsequent transport of proteins from the ER (2). BiP associates transiently with a variety of nascent secretory proteins (3–7) and more stably with malfolded or unassembled proteins whose transport from the ER is blocked (2). BiP is proposed to play a role in protein folding, subunit assembly, and subsequent transport of proteins from the ER (2). BiP associates transiently with a variety of nascent secretory proteins (3–7) and more stably with malfolded or unassembled proteins whose transport from the ER is blocked (2). BiP is proposed to play a role in protein folding, subunit assembly, and subsequent transport of proteins from the ER (2). BiP associates transiently with a variety of nascent secretory proteins (3–7) and more stably with malfolded or unassembled proteins whose transport from the ER is blocked (2).

All HSP70 family members possess a highly conserved NH2-terminal ATP binding domain and a more variable COOH-terminal protein binding domain (10, 11). The three-dimensional structure of the NH2-terminal 44-kDa proteolytic fragment of bovine brain hsc70, a cytoplasmic HSP70, has been determined. This fragment contains the ATP binding site, and its structure is very similar to that of the other adenine nucleotide binding proteins: actin and hexokinase, even though their amino acid sequence homology is not high (12, 13). Studies on both recombinant and purified bovine hsc70 revealed that hsc70 binds ATP and ADP with Keq values in the order of 10−5–10−6 M (14–18). Although all family members presumably have a similar nucleotide binding structure, direct binding studies have not been done on BiP or other HSP70 members. The HSP70s also have a rather weak intrinsic ATPase activity.

In this report, we describe the nucleotide binding properties and ATPase activity of rBiP purified from bacteria. Hamster BiP was tagged with six histidines at the NH2 terminus (6X-His), expressed, and affinity purified on Ni2+-agarose to near homogeneity. By engineering a Factor Xa cleavage site immediately following the histidines and comparing the 6X-His-tagged BiP with the tag-cleaved BiP, we found that the six-histidine tag had no effect on either the structural or ATPase properties. The ATPase activity of rBiP was characterized in terms of pH optimum, salt, and divalent cation requirements, and these properties were essentially the same as those reported for BiP purified from tissue (20). Like hsc70, the rBiP purified from bacteria contained tightly bound ADP, and removal of this nucleotide allowed us to measure the nucleotide binding directly by equilibrium dialysis. These characterization are not only important for establishing the enzymatic parameters of BiP, but will also enable us to identify BiP ATPase and ATP binding mutants that can be used to determine the role of nucleotide in BiP’s function.

MATERIALS AND METHODS

Expression of 6X-His Tagged BiP in Bacteria—A cDNA clone for hamster BiP (21) was ligated into the QE10 vector (Qiagen) in such a way that the leader sequence of BiP was removed and replaced with six histidines and seven non-native amino acids (22). Recombinant hamster BiP protein was expressed in M15 E. coli and purified on Ni2+-agarose. Protein quantitation was done by Bradford’s dye binding assay using bovine serum albumin as a standard (Bio-Rad).

Factor Xa Site Engineering and Subsequent Cleavage of the Histidine Tag—The Factor Xa tetrapeptide recognition site, IEGR, was engineered immediately NH2-terminal of the first amino acid of the mature protein.
hamster rBiP protein (signal sequence-cleaved BiP) and just COOH-
terminal of the six histidines by using a primer extension method (23). A 39-mer oligonucleotide was synthesized that contained a BamHI site, followed by an 1EGR-encoding DNA sequence, and ending with the nucleotides encoding the first six amino acids of mature BIP (CGG GAT CCG ATC GAA GAT AGA GAG GAG GAC AAG). This oligo was used to probe the 5‘ primer, and an 18-base pair oligo flanking Gly260 of BIP was used as the 3‘ primer for amplification by polymerase chain reaction. The polymerase chain reaction-amplified fragment was digested with BamHI and BstEII and cloned into the pTZBiP vector (22) in place of the original BamHI-BstEII fragment. The sequence of the inserted fragment was confirmed by dyelexy sequencing, and the entire BiP insert was then cloned into the pQE10 vector for production of rBiP protein. The resultant protein (His-Xa-BiP) had an NH2-terminal 6His tag followed by the IEGR motif, which is recognized and cleaved by Factor Xa. The Ni2+-agarose purified protein was digested with Factor Xa (U. S. Biochemical Corp.) for 18 h at 30°C with 1:100 (w/w) of Factor Xa to His-Xa-BiP in our modified Xa buffer, containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 2.0 mM CaCl2, and 1 mM sodium azide. We were unable to use the classical Tris containing buffer for Xa digestion, so we used the conserved NH2-terminal region of all HSP70 members. Proteolysis of Recombinant BiP in the Presence of Nucleotide—

Nucleotide Binding and ATPase of rBiP

RESULTS

Engineering of a Factor Xa Site following the Six Histidine Tag and Subsequent Purification of Tag-cleaved rBiP—In a previous report from this laboratory (22), a 6X-His-tag was inserted in place of the BIP signal sequence, which is normally cleaved when BIP is translocated into the ER of mammalian cells. This 6X-His tag allows a simple one-step purification of rBiP. However, it was not determined whether the presence of six positively charged amino acids at the NH2-terminus of the ATP binding domain influences the ATP binding or hydrolysis properties of the tagged-BiP. This concern is particularly valid in order to evaluate any effects of these histidines, a specific protease cleavage site was inserted between the 6X-His tag and the sequence corresponding to the NH2-terminus of the mature BiP protein (Fig. 1A). The Factor Xa recognition tetrapeptide, IEGR, was chosen for the following reasons: 1) this sequence does not exist in the BIP molecule, and 2) cleavage with Xa occurs immediately after the arginine residue in the tetrapeptide, producing a BIP molecule identical in amino acid sequence to mature BIP. The bacterially expressed BIP with 6X-His-IEGR on the NH2-terminus was first purified on a Ni2+-agarose column and subsequently digested with Factor Xa for varying amounts of time (Fig. 1B). The cleavage was nearly complete at 18 h. After rebinding the sample to Ni2+-agarose to remove any remaining uncleaved BiP, the Xa-cleaved protein is quite pure (Fig. 1B, purified). Isolation of pure BiP protein containing the 6X-His tag, as well as protein devoid of the His tag, allowed us to determine whether the His tag affected either structural or ATPase properties of rBiP.

Oligomerization and Conformation of Tag Cleaved as Well as Uncleaved rBiP—In mammalian cells, BIP exists as both a dimer and monomer (25). Nondenaturing gel electrophoresis was used to analyze the oligomerization status of the tag-cleaved and uncleaved forms of rBiP. Both of the 6X-His-tagged

Fig. 1. Engineering of a Factor Xa cleavage site following the six-histidine tag on rBiP and purification of tag-cleaved rBiP (mature BIP). A, NH2-terminal amino acid sequence alignment of dndk, hsc70, and BIP. The locations of His tag and Factor Xa recognition site on BIP constructs are underlined. The boxed sequences denote the conserved NH2-terminal region of all HSP70 members. B, affinity purified His-Xa-BiP was digested with Factor Xa for different time periods and an aliquot of each was analyzed on SDS-PAGE. The 18-h sample was reincubated with Ni2+-agarose to remove the remaining His-tagged BiP and obtain purified mature BIP.

| Time (h) | Cleaved |
|---------|---------|
| 0       |         |
| 4       |         |
| 8       |         |
| 12      |         |
| 16      |         |

Nucleotide Binding and ATPase—

HPLC Analysis of BIP Nucleotide Content and Preparing Nucleotide-free BIP—The protocol of Gao et al. (18, 27) was used to determine the nucleotide content of rBiP and to prepare nucleotide-free protein. For nucleotide determinations, 10 μM rBiP was acid denatured and then precipitated to separate protein from released nucleotide. The sample was neutralized with KOH and centrifuged by centrifugation before applying it to the HPLC column. An HPLC system consisting of a Pharmacia LKB 2122 pump (22) equipped with a Pharmacia LKB 2141 monitor, and a LKB 2221 integrator was used. The nucleotide-free BIP was prepared by incubating purified rBiP with a 600-fold molar excess of AMPNP to replace the bound nucleotide and then dialyzing it in the standard ATPase buffer. The extent of nucleotide removal was determined by HPLC.

Nucleotide Binding—Nucleotide binding was performed by equilibrium dialysis as described by Gao et al. (27). These experiments, cylindrical 200-μl volume dialysis chambers filled with dialysis membranes (MWCO 25 kDa) were used. 0.3 mg/ml bovine serum albumin was added to each chamber and the samples were dialyzed against dialysis buffer with 2.0 μM rBiP on one side and 0.25–4.0 μM 14C]ATP or ADP (with constant specific activity) on both sides of the dialysis membrane. The dialysis chamber was rotated at 4°C with a speed of 10 rpm and
proteins (His-Xa-BiP and His-BiP) and the Xa-cleaved BiP (mature) existed as a combination of monomers, dimers, and some higher order oligomers (Fig. 2A), suggesting that the histidine tag did not grossly alter the structure of the rBiP proteins. The Xa-cleaved BiP migrates slightly faster on non-denaturing gels, presumably due to the removal of the positivelycharged peptide. The migration patterns of the three purified rBiP proteins are nearly identical to each other and to that observed when purified mammalian BiP and hsc70 were similarly analyzed (28, 29).

Partial protease digestion is often used as a measure of the structural integrity of a protein. The HSP70 proteins produce very distinctive proteolytic patterns when digested in the presence of nucleotides. ATP protects 60- and 44-kDa fragments, whereas, ADP protects only the 44-kDa fragment (11, 20). In order to check the structural integrity of our three forms of rBiP, we digested the various samples with proteinase K, a nonspecific serine protease, in the presence of ATP or ADP. For all three of the BiP preparations, both a 60- and a 44-kDa fragment were protected when ADP or no nucleotide was present (Fig. 2B). These protected fragmentsof the three purified rBiP proteins are nearly identical to each other and to that observed when purified mammalian BiP and hsc70 were similarly analyzed (28, 29).

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Characterization of the ATPase Activity of rBiP and Comparison of Tag Cleaved versus Uncleaved rBiP in Terms of ATPase Activity—Knowing that our recombinant proteins maintained the structural properties of endogenous BiP, we next wished to examine the ATPase activity of these rBiP proteins. In this study, an ATPase assay measuring the release of inorganic phosphate was chosen (26). This method is sensitive, easy to perform, and suitable for measuring ATP hydrolysis across a wide range of ATP concentrations. The ATPase activities of the three different forms of rBiP were compared under identical conditions (Fig. 3A). For this experiment, His-Xa-BiP was mock-digested to control for any effects of the digestion conditions. Interestingly, we found that mock-digested BiP had identical ATPase activity when compared to His-Xa-BiP that was kept at 4 °C, indicating that BiP is fairly stable at 30 °C for at least 18 h (not shown). The ATPase activity of the cleaved rBiP (mature BiP) was essentially identical to both of the tagged forms of BiP (Fig. 3A).

The pH effect on BiP’s ATPase activity was examined by using the same buffers described for assaying endogenous BiP (20). Peak ATPase activity was obtained at pH 5.0 while either increasing or decreasing the pH from pH 5.0 significantly lowered the ATPase activity (Fig. 3B). For all further ATPase determinations, pH 7.0 was used because: 1) the ER pH is estimated to be approximately pH 7.0; 2) pH 7.0 is in a region of the pH curve with a shallow slope, thus minimizing the potential for large measurement errors; and 3) the ATPase and ATP binding activities of hsc70 were analyzed at pH 7.0 (14, 18, 30). Thus, by performing our ATPase assays at pH 7.0, it will be possible to compare findings between these two related proteins.

We next assayed the effects of salt and divalent cations on BiP’s ATPase activity. The data for cleaved and His-tagged BiP were indistinguishable, and thus, for simplicity, only that for cleaved BiP (mature) is presented. The optimal salt concentration was about 25 mM for both NaCl and KCl, but at all concentrations measured, KCl produced a higher ATPase activity than NaCl (Fig. 3C). Therefore, all remaining assays were performed with 25 mM KCl. Calcium was strongly inhibitory to BiP’s ATPase activity, and magnesium was required for the optimal enzyme activity (Fig. 3D). This is consistent with the requirements for both native BiP and hsc70 (20, 30), but unlike data on hsc70, Mg(CH₂CO₂)₂ was not preferred over MgCl₂. The optimal concentration of MgCl₂ required for rBiP’s ATPase activity ranged from between 1 and 10 mM. The ATPase activity of rBiP could be stimulated 2-3-fold by peptides (see Ref. 43, accompanying article), which is similar to data for native mammalian BiP and hsc70 (28, 31). Taken together, these data demonstrate that the ATPase properties of our rBiP purified from bacteria are extremely similar to those of BiP purified from dog pancreas (20). In no case was there a significant difference between 6X-His-tagged and tag-cleaved BiP, demonstrating that the 6X-His tag does not affect any of the various aspects of BiP’s ATPase activity. In the following study (43), 6X-His BiP was used in order to avoid additional manipulations to the recombinant proteins during purification.

Nucleotide Content of rBiP and Preparation of Nucleotide-free rBiP—The inability of our rBiP to bind nucleotide in equilibrium dialysis experiments alerted us to the possibility that the rBiP purified on Ni²⁺-agarose might contain bound nucleotide, even though our rBiP was not purified on ATP-agarose.
If this was the case, the nucleotide must have bound in the bacteria and must be resistant to dialysis, since the BiP was dialyzed before the binding study. To determine if our rBiP contained bound nucleotide, the recombinant protein was-perchloric acid-denatured and precipitated. The supernatant, which should contain any released nucleotides, was analyzed by HPLC. The data showed that 0.82 mol of ADP was bound per mol of rBiP (Fig. 4A). The absence of ATP in this BiP preparation was not due to hydrolysis during the extraction procedure, since no hydrolysis occurred when an ATP standard was treated in parallel (data not shown).

At this point it was necessary to prepare nucleotide-free BiP in order to measure the nucleotide binding constants for ATP and ADP. We used the method developed for preparing nucleotide-free hsc70 (18). A 600-fold molar excess of AMPPNP was added to rBiP, the sample was incubated at room temperature for 1 h to replace the bound ADP, and then the bound AMPPNP, as well as free nucleotides, were removed by dialysis. Over 95% of the bound ADP was removed after this treatment, as shown by HPLC analysis (Fig. 4). The nucleotide-free BiP was stable for about 2 weeks when kept at 4°C as determined by measuring ATPase activity (not shown). The oligomerization state and protease digestion patterns were not altered by removing the nucleotide, suggesting that ADP is not required for BiP oligomerization and that nucleotide-free BiP is structurally similar to BiP containing bound ADP (not shown).

**Kinetic Properties of rBiP**—The kinetic properties of the nucleotide-free rBiP's ATPase activity were examined using the optimized conditions derived from the initial ATPase characterization. In this experiment, several enzymatic criteria were strictly observed. First, the enzyme concentration used was in the linear region in the initial velocity versus [rBiP] plot (Fig. 5A). Second, the lowest substrate (ATP) concentration was at least 10-fold greater than the enzyme concentration. And third, at the lowest substrate concentration for a given amount of enzyme, the incubation time was chosen such that it was in the linear portion of the time course curve (Fig. 5B). Under standard assay conditions and using a constant amount of enzyme (0.05 mM), ATP concentrations from 0.5 to 6.0 mM were used for assaying the ATPase activity (Fig. 5C). The initial velocities obtained and the corresponding ATP concentrations were entered into the Cleland's kinetic program that determines the $V_{max}$ and $K_m$ values based on a Lineweaver-Burk's plot (Fig. 5D). A $V_{max}$ of 5.2 pmol/min/μg and a $K_m$ of 1.5 mM were obtained. The $V_{max}$ corresponds to a $k_{cat}$ of 0.40 min$^{-1}$ and is consistent with the slow rate of hydrolysis reported for native BiP and other HSP70 family members.

**Equilibrium Dialysis Measuring Nucleotide Binding of rBiP**—Equilibrium dialysis was used to measure the dissociation constants of nucleotide-free rBiP for ADP and ATP. With increasing ADP concentrations, the portion of bound nucleotide increased until it approached saturation at 4 μM (Fig. 6A).
that was identical in sequence to mature hamster BiP isolated of the 6X-His tag so it could be removed, producing a protein engineered a factor Xa cleavage site immediately downstream amino acid to the ATP binding domain that could affect kinetic but unlike native BiP, resulted in the addition of non-native terminus allowed easy purification of the rBiP from bacteria, protein. Tagging hamster BiP with six histidines at its amino isolate mutant proteins that does not require ATP binding or the normal kinetic values for wild-type BiP, and a method to ing and ATP hydrolysis mutants requires both a knowledge of Further increases in the ADP concentration up to 200 μM did not increase the amount of nucleotide bound to BiP (not shown). For the ATP binding measurement, creatine kinase and creatine phosphate were included in both chambers since a significant amount of the ATP was hydrolyzed if the ATP regenerating system was not included (not shown). The efficiency of creatine kinase and creatine phosphate to convert ADP to ATP was almost 100%, as demonstrated by thin layer chromatography performed at the end of dialysis (not shown). The ADP and ATP binding data were replotted in a Scatchard plot (Fig. 6, A and B, insets), and the resultant number of binding sites ranged from 0.7 to 1.1 for both ATP and ADP depending on different preparations. The $K_d$ values of 0.2 ± 0.1 and 0.29 ± 0.12 μM were obtained for ATP and ADP, respectively. However, within a single protein preparation, the variations were small (Fig. 6). The stability of BiP under these binding conditions was examined by measuring the amount of bound nucleotide at both 24 h (time required to reach equilibrium) and at 48 h. There was no loss of bound nucleotide even after 48 h of dialysis, suggesting that rBiP was stable under the binding conditions used.

**Fig. 4. HPLC analysis of nucleotide bound to rBiP.** a, buffer alone was extracted as a control and the background profile was determined. b, nucleotide was extracted from 10 μM rBiP as described under "Materials and Methods.” c, rBiP was prenublated with 600 molar excess of AMPNPN to replace bound nucleotide and then extensively dialyzed to remove both bound and free nucleotides. Nucleotide was extracted as in b. A control sample containing a mixture of ADP, AMPNPN, and ATP was injected to determine where each of them eluted and each is denoted with a downward arrow.

Further increases in the ADP concentration up to 200 μM did not increase the amount of nucleotide bound to BiP (not shown). For the ATP binding measurement, creatine kinase and creatine phosphate were included in both chambers since a significant amount of the ATP was hydrolyzed if the ATP regenerating system was not included (not shown). The efficiency of creatine kinase and creatine phosphate to convert ADP to ATP was almost 100%, as demonstrated by thin layer chromatography performed at the end of dialysis (not shown). The ADP and ATP binding data were replotted in a Scatchard plot (Fig. 6, A and B, insets), and the resultant number of binding sites ranged from 0.7 to 1.1 for both ATP and ADP depending on different preparations. The $K_d$ values of 0.2 ± 0.1 and 0.29 ± 0.12 μM were obtained for ATP and ADP, respectively. However, within a single protein preparation, the variations were small (Fig. 6). The stability of BiP under these binding conditions was examined by measuring the amount of bound nucleotide at both 24 h (time required to reach equilibrium) and at 48 h. There was no loss of bound nucleotide even after 48 h of dialysis, suggesting that rBiP was stable under the binding conditions used.

**DISCUSSION**

The ability to identify and fully characterize BiP ATP binding and ATP hydrolysis mutants requires both a knowledge of the normal kinetic values for wild-type BiP, and a method to isolate mutant proteins that does not require ATP binding or that does not result in co-contamination with wild-type BiP protein. Tagging hamster BiP with six histidines at its amino terminus allowed easy purification of the rBiP from bacteria, but unlike native BiP, resulted in the addition of non-native amino acids to the ATP binding domain that could affect kinetic measurements. To determine if the 6X-His tag affected BiP, we engineered a factor Xa cleavage site immediately downstream of the 6X-His tag so it could be removed, producing a protein that was identical in sequence to mature hamster BiP isolated from cells.

The 6X-His tagged and the tag-cleaved rBiP seemed to be structurally identical to native mammalian BiP as judged by protease protection assays (20) and by their ability to form dimers and monomers (25, 28). Similar to other studies performed on native BiP (29, 31), we found that the dimers were converted to monomers by peptide binding (not shown), and that the ATPase activity was stimulated approximately 2-3-fold by peptide (Ref. 43, accompanying paper). This is in contrast to another report, in which rBiP was isolated, in part, on ATP-agarose columns (29). Their rBiP was primarily monomeric and was not stimulated by peptide. We found that all three forms of our rBiP had the same rates of ATP hydrolysis, and that their pH and salt optima, as well as the divalent cation requirements for this activity were the same as those reported for native BiP (20). Thus, our purification method produced rBiP proteins that appeared to be structurally very similar to native BiP, and the 6X-His tag did not apparently affect structural or enzymatic parameters.

Our recombinant BiP possessed tightly bound ADP, just like native hsc70 isolated from bovine brain (14, 27). Unlike the purification scheme for bovine hsc70 (14, 27), we did not use nucleotide columns and ATP elution in our isolation. Therefore, the bound ADP must come from the bacteria and is most likely a normal component of HSP70 proteins. After producing nucleotide-free BiP, we performed ATPase assays to determine the $K_m$ (1.48 ± 0.1 μM) and $V_{max}$ (5.12 pmol/min/μg) of wild-type BiP. The variations between reported values for $V_{max}$ of HSP70 proteins are surprisingly large. The $V_{max}$ values for BiP range from 0.2 to 4.7 (19, 20), for hsc70 from 1.2 to 12 (32, 33), and for dnak from 2.5 to 600 pmol/min/μg (34, 35). This variation could stem from the fact that, in most studies, the percent of the preparation that was active is not known, no attempt was made to remove bound nucleotide before performing the assays, and finally, the purity of the various preparations used in these studies is not known. Because the HSP70s are very poor ATPases, even a small amount of contamination with a much stronger ATPase could have profound effects on the $V_{max}$ value. We feel that our determination of $V_{max}$ and $K_m$ values are reliable, because we ensured that our protein possessed nearly full ATP binding activity and was free of nucleotide before performing kinetic studies. We determined that our preparation was free of contamination by immunoblotting with anti-dnak antisera and by performing in vitro kinase assays (data not shown). The purity of our rBiP preparation was further corroborated by our isolation of an ATPase mutant, T229G BiP (43) that was purified in the same way. Finally, care was taken to ensure that our studies followed strict enzymatic criteria.

There is currently no reported data on direct nucleotide binding for BiP, however, there are reports for hsc70 prepared either from bovine brain or bacteria with $K_d$ values for ADP or ATP ranging from $10^{-5}$ to $10^{-8}$ M. Using initial rate binding, Schmid et al. (14) measured nucleotide binding activities of bovine hsc70 and reported a $K_d$ of 1.35 μM for Mg-ADP and a $K_d$ of 0.7 μM for Mg-ATP, and found that there was 0.4 mol of nucleotide/mol of protein that was not removed by dialysis. Using equilibrium dialysis on hsc70, Palleros et al. (15) measured a $K_d$ of 1.6 μM for Mg-ADP and a $K_d$ of 9.5 μM for Mg-ATP (15). These weaker binding affinities, as compared to other reported values may be due to the presence of bound nucleotides in the preparations which could distort the binding data. Using nucleotide-free recombinant hsc70, Wang and Lee (16) reported a $K_d$ of 0.2–0.3 μM for Mg-ATP (16), and Ha and McKay (17) reported a $K_d$ of 0.042 for Mg-ATP and 0.11 μM for Mg-ADP. The values for recombinant hsc70 are close to our measurements for recombinant BiP. Using AMPNPN as a primary binder in a competition study, Gao et al. (18) measured a
of 0.012 M for Mg-ATP and 0.018 M for Mg-ADP for nucleotide-free hsc70 purified from bovine brain. These values are somewhat lower than those for recombinant hsc70 or BiP, and the discrepancy may result either from differences in the experimental methods used, or from an intrinsic difference between native and recombinant proteins.

The weak ATPase activity and high ATP binding affinity of BiP measured in vitro suggests one of two things. First, other ATPase activating proteins may exist. In bacteria, two heat shock proteins, dnaJ and grpE, stimulate the ATPase activity of dnaK up to 50-fold (36). Cytosolic homologues of dnaJ have been identified in human and yeast cells (37–40), and an ER protein with homology to dnaJ has been isolated in yeast (41). However, no data are available to demonstrate that these proteins modulated the ATPase activity of eukaryotic HSP70 proteins. Alternatively, ATP binding may be more important to the function of the HSP70 proteins than ATP hydrolysis. This may be particularly true for BiP since the assumed ER conditions, pH 7.4 and millimolar calcium, strongly inhibit BiP's ATPase activity. An in vitro study using purified proteins demonstrated that ATP binding, but not ATP hydrolysis, was required for the release of bound polypeptides from hsp70 and dnaK (42, 43).
In summary, we have isolated recombinant BiP protein from bacteria and demonstrated that enzymatically it behaves very much like native BiP isolated from mammalian cells. Using rBiP purified by a single step, we determined the $K_m$ and $V_{\text{max}}$ values for wild-type BiP and provided the first determination of nucleotide binding affinities for BiP. The characterization of wild-type BiP activity permits us to analyze the various BiP ATPase mutants (43) to determine whether they represent nucleotide binding or hydrolysis mutants. This should then allow us to distinguish the role of ATP binding from that of ATP hydrolysis in BiP function.

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