Mammalian alkaline phosphatases (APs) display 10–100-fold higher $k_{\text{cat}}$ values than do bacterial APs. To begin uncovering the critical residues that determine the catalytic efficiency of mammalian APs, we have compared the sequence of two bovine intestinal APs, i.e. a moderately active isozyme (bovine intestinal alkaline phosphatase, bIAP I, $\sim 3,000$ units/mg) previously cloned in our laboratory, and a highly active isozyme (bIAP II, $\sim 8,000$ units/mg) of hitherto unknown sequence. An unprecedented level of complexity was revealed for the bovine AP family of genes during our attempts to clone the bIAP II cDNA from cow intestinal RNAs. We cloned and characterized two novel full-length IAP cDNAs (bIAP III and bIAP IV) and obtained partial sequences for three other IAP cDNAs (bIAP V, VI, and VII). Moreover, we identified and partially cloned a gene coding for a second tissue nonspecific AP (TNAP-2). However, the cDNA for bIAP II, appeared unclonable. The sequence of the entire bIAP II isozyme was determined instead by a classical protein sequencing strategy using trypsin, carboxypeptidase, and endoproteinase Lys-C, Asp-N, and Glu-C digestions, as well as cyanogen bromide cleavage and NH$_2$-terminal sequencing. A chimeric bIAP II cDNA was then constructed by ligating wild-type and mutagenized fragments of bIAP I, III, and IV to build a cDNA encoding the identified bIAP II sequence. Expression and enzymatic characterization of the recombiant bIAP I, II, III, and IV isozymes revealed average $k_{\text{cat}}$ values of 1800, 5900, 4200, and 6100 s$^{-1}$, respectively. Comparison of the bIAP I and bIAP II sequences identified 24 amino acid positions as likely candidates to explain differences in $k_{\text{cat}}$. Site-directed mutagenesis and kinetic studies revealed that a D322G mutation in bIAP II reduced its $k_{\text{cat}}$ to 1300 s$^{-1}$, while the converse mutation, i.e. D322G, in bIAP I increased its $k_{\text{cat}}$ to 5800 s$^{-1}$. Other mutations in bIAP II had no effect on its kinetic properties. Our data clearly indicate that residue 322 is the major determinant of the high catalytic turnover in bovine IAPs. This residue is not directly involved in the mechanism of catalysis but is spatially sufficiently close to the active site to influence substrate positioning and hydrolysis of the phosphoenzyme complex.

**Active Bovine Intestinal Alkaline Phosphatases**

Mammalian APs display the unique kinetic property, not shared by their bacterial ancestors, of being inhibited sterically by $L$-amino acids and peptides through an uncompetitive mechanism (7, 8). Using human placental AP as a paradigm for mammalian APs, we and others have established that residues within a surface loop unique to mammalian APs are responsible for the differential uncompetitive inhibition by $L$-amino acids (9–12), the heat stability properties (13), and protein-protein interaction specificities exhibited by some mammalian APs (13). A major property of APs that remains to be explained in terms of structure is the large variability in catalytic activity displayed by mammalian APs, which have 10–100-fold higher $k_{\text{cat}}$ values than E. coli AP (14). Because among mammalian APs, the intestinal isozyme has the highest specific activity, the bovine intestinal APs (IAPs) represent a potentially useful system for addressing this question. Besman and Coleman (15) demonstrated the existence of two IAP isozymes in the cow intestine, i.e. calf IAP and adult bovine IAP, by sequencing the amino termini of chromatographically purified AP fractions. We have previously reported the cloning and biochemical characterization of the recombinant adult bovine IAP, presently designated bIAP I (16). In this study we report the sequence and characterization of the calf IAP (bIAP II) and two novel bIAP isozymes (bIAP III and bIAP IV (GenBank™ accession nos. AF052226 and AF052277)) and present evidence for the existence of an unprecedented level of complexity in the cow AP gene family. Sequence comparisons and

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1 The abbreviations used are: AP, alkaline phosphatase; IAP, intestinal alkaline phosphatase; bIAP I–VII, bovine intestinal alkaline phosphatase I–VII isozymes; TNAP, tissue nonspecific alkaline phosphatase; TNAP-2: tissue nonspecific alkaline phosphatase II isozyme; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; pNPP, $p$-nitrophenylphosphate; GPI, glycosylphosphatidylinositol; bp, base pair(s).
Materials and Methods

Cloning—A 14 nt 11 cDNA library prepared from adult cow intestine (CLONTECH Laboratories, Palo Alto, CA) was screened using a 1,075-bp HindIII fragment from the 5’ end of the bIAP I cDNA (16) as a probe. Clones isolated from this library were used to screen an EMBL-3 SP6/T7 genomic library prepared from adult cow’s liver (CLONTECH Laboratories). An unamplified ZAP II cDNA library was prepared from mRNA isolated, using the Trisolv™ reagent, from the EMBL-3 SP6/T7 genomic library prepared from adult cow’s liver as a probe. Clones isolated from this library were used to screen an adult cow genomic library (CLONTECH Laboratories, Palo Alto, CA) was screened using a 492A protein sequencer (Applied Biosystems, Weiterstadt) ac-

Construction of bIAP II cDNA—To construct a cDNA coding for bIAP II, wild-type restriction fragments and site-directed mutated gene PCR fragments of cDNAs bIAP I, III, and IV were assembled, creating the LIN8 (three fragments) and INT 1 (nine fragments) intermediate cDNA constructs. INT 1 and bIAP III then served as the templates for site-directed mutagenesis and fragments from these were assembled into a complete INT 2 (eight fragments) cDNA. Restriction fragments from INT 2 and site-directed mutated fragments of INT 2 were then assembled into the INT 3 (five fragments) cDNA and finally the bIAP II construct (cDNA) cDNA. Site-directed mutagenized mutants as described previously (19) using BsaI as the restriction enzyme that cuts at a distance from its recognition site (GGTTC/TCN/NS). All PCR products were sequenced to verify the absence of secondary mutations, and all constructs were verified by sequencing and restriction digestion. The sequence of the oligonucleotide primers used for amplifying the site-

Determination of the Amino Acid Sequence of bIAP II—Approximately 500 μg of purified, highly active (approximately 7800 units/mg) cGST were dissolved in 50 μl of lysis buffer (250 mM NaHPO4, 50 mM EDTA, pH 7.2), and 15 units of endoglycosidase F (Boehringer Mannheim, Penzberg). The mixture was left overnight at 37 °C and subsequently used for cleavages. Reduced and alkylated AP was enzymatically cleaved with different enzymes (i.e. endoproteinase Lys-C, endoproteinase Asp-N, endoproteinase Glu-C, and trypsin (Boehringer Mannheim, Penzberg) according to the instructions given on the data sheets for individual enzymes. Cyanogen bromide cleavage was performed with 10% (w/v) CNBr in 70% (v/v) formic acid for 8 h. After dilution with water the solution was reduced in volume on a SpeedVac concentrator (Savant) and applied onto reverse phase HPLC. Carboxypeptidase Y (8 ng/mg) digestion of the COOH-terminal tryptic peptide was performed for 4 min, and the re-

L1N8 or bIAP I (I) and bIAP II (II). L1N8 (three fragments) and INT 1 (nine fragments) intermediate cDNA constructs. INT 1 and bIAP III then served as the templates for site-directed mutagenesis and fragments from these were assembled into a complete INT 2 (eight fragments) cDNA. Restriction fragments from INT 2 and site-directed mutated fragments of INT 2 were then assembled into the INT 3 (five fragments) cDNA and finally the bIAP II construct (cDNA) cDNA. Site-directed mutagenized mutants as described previously (19) using BsaI as the restriction enzyme that cuts at a distance from its recognition site (GGTTC/TCN/NS). All PCR products were sequenced to verify the absence of secondary mutations, and all constructs were verified by sequencing and restriction digestion. The sequence of the oligonucleotide primers used for amplifying the site-

site-directed mutagenesis have unequivocally identified a Gly residue at position 322 as the crucial residue that determines the high specific activity of bIAP II.
E210V +, GGT CTC ATG TTT CCT GTG GGG ACC CCA GAC; E236A, GGT CTC CCT CCA TGC CTG CAC CAG GTT. Using these and previously listed oligos the following eight PCR reactions (a–h) were carried out using bIAP II as template: a, 1s, 1133M−; b, S142A−, M205K−; c, 1s, A142S−; d, V210E +, 330−; e, E210V +, 330−; f, M180K +, E236A−; g, 336 +, 330−; h, S142A, K205M−. The products of these were subcloned and sequenced, and then fragmented isolated for the following ligations: (II) EcoRI-NcoI, (a) NcoI-BstI, (b) BstI-PvuII, (II) PvuII-XbaI for 1133M. (II) EcoRI-EcoRI, (c) NcoI-BstBII, (II) BstBII-PvuII, (II) PvuII-XbaI for A142S. (II) EcoRI-BstBII, (b) BstBII-BstI, (d) BstI-HindIII, (II) HindIII-XbaI for M205K. (II) EcoRI-BstBII, (b) BstBII-BstI, (e) BstI-HindIII, (II) HindIII-XbaI for E210V. (II) EcoRI-NcoI, (f) NcoI-PvuII, (f) PvuII-BstI, (g) BstI-HindIII, (II) HindIII-XbaI for E236A.

Production and Characterization of the Recombinant Enzymes—All cDNAs (bIAP I, bIAP II, bIAP III, bIAP IV, and corresponding mutants) were cloned into the pcDNA-3 expression vector (Invitrogen, San Diego, CA), transfected into Chinese hamster ovary cells and stable transfectants were selected by growing the cells in the presence of 50 μg/ml geneticin (Life Technologies, Inc.). Recombinant APs were extracted from the stably transfected Chinese hamster ovary cells as described previously (20). To measure kcat, microtiter plates coated with 0.1 μg/ml high affinity anti-bovine AP monoclonal antibody (Scottish Antibody Production Unit, Lanarkshire, Scotland) were incubated with increasing concentrations of enzyme and the activity of bound enzyme was measured as the change in absorbance at 405 nm over time at 20 °C. The residual activity remained (compared with the nonheated sample. The temperature at which 50% above, and residual activity calculated as the percentage remaining.

RESULTS AND DISCUSSION

Cloning of bIAP III and IV—We set out to determine the structure of the fetal intestinal AP (bIAP II) defined by Besman and Coleman (15) as possessing a LIPAEEEN amino-terminal sequence since we knew that this amino-terminal sequence was found in purified high activity intestinal AP preparations (range 7,000 to 8,000 units/mg) available commercially (Biozyme Laboratories and Boehringer Mannheim GmbH). We screened a commercial 5’ Stretch Agt 11 bovine small intestinal cDNA library (CLONTECH Laboratories) with a 1,075-bp HindIII fragment of the bIAP I cDNA that contains sequences included in exons I through VIII of the bIAP I gene. Twelve cDNAs were isolated that represented different size spots and intensity of hybridization on the filters. The fragments were subcloned and completely sequenced. Four of these cDNAs were identical to the bIAP I sequence as previously published (16). Four clones represent a new tissue-specific AP isozyme gene homologous, but not identical, to bIAP I, although the clones were unspliced. The largest of these clones (2,561 bp) aligned to the bIAP I gene 570 bp 5’ of the start codon and extended to exon eight, and identifies the isozyme referred to here as bIAP VII (GenBank™ accession no. AF052230). Two cDNAs represented another variation of the bIAP I sequence and represented unspliced clones, the largest (783 bp) contained exon I to exon III sequences which define the bIAP V isozyme (GenBank™ accession no. AF052228). One cDNA (clone VIII) appeared to be yet another tissue-specific AP transcript. This clone is 1,642 bp long, is also unspliced, aligns with bIAP I from intron 2 to intron 8, and defines the bIAP VI isozyme GenBank™ accession no. AF052229). All these novel clones encode predicted amino-terminal sequences that are different from the expected LIPAEEEN sequence.

Fig. 1 shows differences in the deduced first 80 amino-terminal amino acids of the newly identified isozymes bIAP III, bIAP IV, bIAP V, bIAP VI, bIAP VII in comparison with the corresponding residues of bIAP I (16) and of bIAP II as determined below. Still another cDNA was isolated that represents a new tissue nonspecific AP molecule. This partially spliced cDNA clone aligns with bovine kidney AP (21) (starting at residue 8) in exon II and extends to exon IX. This appears to be a different TNAP (TNAP-2, GenBank™ accession no. AF052231) molecule expressed in the bovine intestine. Northern blot analysis was performed on RNA samples isolated from different portions of the cow intestine of a single animal, and the sample with the highest expression was chosen for the construction of a new cDNA library in azAP II vector (Stratagene). Adjacent segments of the intestine were used for enzyme purification. The entire unamplified library (1.0 × 106 independent recombinant clones) was screened with the 1,075-bp HindIII bIAP I probe, and 65 clones were isolated and sequenced. All clones corresponded in sequence to one or the other of two novel bIAP cDNAs designated bIAP III and bIAP IV. The sequence of the 2,460-bp bIAP III cDNA is shown in Fig. 2 as well as the differences found in the coding region of the 2,536-bp bIAP IV cDNA. Neither of these full-length novel bIAP cDNAs coded for an amino-terminal LIPAEEEN sequence, while at the protein level it was clear that the LI-PAEEEN sequence was the major component of the purified preparation from the same intestinal region. We had to conclude that the bIAP II sequence was either "toxic" to the bacterial cells used for library construction or "unclonable" for some other reason.

Our previous work on the cloning of bIAP had revealed the structure of the bIAP I gene and also of a transcribed pseudogene (R201) (16). Southern blot analysis using the bIAP cDNA as a probe had revealed a complex pattern of bands and only two of them could be accounted for by the cloned bIAP I gene and the R201 pseudogene (16). Human and mouse APs are the two best characterized gene families, and both display the
FIG. 2. Complete nucleotide sequence of the 2,460-bp bIAP III cDNA and deduced amino acid sequence. Nucleotide differences found in the coding region of the bIAP IV cDNA are written above the nucleotide sequence and those mutations that translate into amino acid differences are spelled out under the deduced amino acid sequence of bIAP III. Nucleotide differences in the 5'- and 3'-untranslated regions of the bIAP III and bIAP IV cDNA are not shown.
same degree of genetic complexity. Human APs are encoded by three tissue-specific AP loci, i.e. GCAP (22), PLAP (23), and IAP (24) and one TNAP locus (25), and the mouse AP genes include two active tissue-specific AP genes, i.e. embryonic and IAP (26), one pseudogene (26), and one TNAP gene (27). Both the human and mouse tissue-specific genes are highly homologous and are each comprised of 11 exons contained in less than 5 kilobase pairs of DNA while the single TNAP gene in both species is composed of 12 exons occupying 40–50 kilobase pairs of genomic DNA. The rat AP gene family has not been as well characterized, although a TNAP gene (28) and two different IAP cDNAs (29), both coding for glycosylphosphatidylinositol (GPI) anchored isozymes, have been cloned. The presence of at least seven IAP genes in the cow intestine and the existence of two tissue nonspecific AP loci in this species is, therefore, unprecedented. Since the cow has also been shown to have genes encoding at least two embryonic AP isozymes (30) the number of AP genes in this species is likely to be 10 or more.

**Determination of the Amino Acid Sequence of the Highly Active bIAP II Isozyme**—We resorted to determining the amino acid sequence of bIAP II using purified commercial preparations of high activity calf intestinal AP (Biozyme AP and Boehringer Mannheim GmbH). Direct NH₂-terminal analysis revealed that purified AP preparations were heterogeneous. The two-dimensional electrophoresis pattern was far more complex than expected for a purified preparation and varied between the different AP preparations (data not shown). After deglycosylation, the two-dimensional gel electrophoresis complexity of the protein pattern remained almost unchanged, suggesting that the observed heterogeneity was mainly caused by differences in the primary structure of the APs, likely due to heterogeneity in the purified AP preparations, rather than by different glycostructures. Peptide maps were generated from the most active AP preparation by cleavage with endoproteinase Lys-C, Asp-N, Glu-C, trypsin, and cyanogen bromide after reduction and alkylation using both native and deglycosylated enzyme. The generated peptides were separated and isolated by reversed phase HPLC. Electrospray mass analysis of each fraction was performed, and the peptides were sequenced by Edman degradation and compared with our previously published amino acid sequence of bIAP I (16). The complete primary structure of bIAP II is given in Fig. 3. As expected, this isozyme possessed the LIPAEEEN amino-terminal sequence. Several unusual cleavages were observed for trypsin at position Tyr²²⁹ and Phe⁴⁶⁴, for endoproteinase Lys-C at position Arg³⁸⁴ and Arg⁴⁴² and for endoproteinase Asp-N at position Glu⁴⁴⁴. Heterogeneities were observed by the appearance of two amino acids almost in the same order of magnitude in positions 205

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**Fig. 3.** Protein sequencing strategy used to determine the amino acid sequence of bIAP II.
(K/M), 251 (T/M), 252 (A/K), 380 (S/G) and in position 133 where the main isoleucine was accompanied by a low amount of methionine (less than 20%). In position 210, the main glutamic acid was accompanied by a low amount of valine (less than 20%). In position 410 no phenylthiohydantoin amino acid residue could be detected in the Edman degradation of Asp-N cleavage derived peptide (384–414), indicating a glycosylation of this position. The peptide (387–420) derived from the Lys-C digest of the deglycosylated material shows in position 410 an aspartic acid. In position 122 no amino acid could be detected, except in the case of peptides derived from deglycosylated material, where an aspartic acid was observed in this position. Thus, the high activity bIAP II isozyme was positively identified as being glycosylated at position 122 and 410, while glycosylation at position 249 remains a possibility. It should be noted that the double signals at positions 133, 205, 210 and 380 are compatible with the presence in the mixture of bIAP II together with either bIAP I, or IV, but given the fact that the starting APs preparation had a very high specific activity, it is most likely that the double signal is contributed by bIAP IV. The double signal at positions 251 and 252 cannot be explained by bIAP I, III, or IV and there is not enough sequence information to determine whether bIAP V, VI, or VII or still other isozymes may also be present.

The site of attachment of the GPI anchor has only been identified in the case of human PLAP to be Asp484 (31, 32). The cDNA of PLAP encodes a sequence of 29 amino acid downstream from Asp484. The sequence of this COOH-terminal peptide varies greatly between GPI anchored proteins (33). Even within the AP isozyme family, this COOH-terminal region, as deduced from cDNA cloning, has the least amount of sequence conservation (2). In the case of bIAPs, the deduced COOH-terminal sequences of bIAP I, III, and IV are considerably similar, so that it can be predicted that all these isozymes would be anchored through an equivalent amino acid position. Previously, we have shown that recombinant bIAP I is GPI-anchored and it can be extracted either as a membrane-bound form retaining its GPI anchor or as a soluble isozyme (16). Our present work suggests that the GPI anchor site may be Ala480 in the case of bIAP II since this was the COOH-terminal amino acid detected in mature bIAP II. This would suggest that bIAP I may be GPI anchored at Thr480 and bIAP III and IV at the corresponding Ser480.

Construction of a Chimeric bIAP II cDNA, Expression and Characterization of the Recombinant bIAP I, II, III, and IV Isozymes—The availability of the bIAP I, III, and IV cDNAs and the primary amino acid sequence of bIAP II made it possible to devise a scheme to construct a chimeric bIAP II cDNA to enable the expression and characterization of the recombinant enzyme. The strategy, depicted in Fig. 4, entailed ligating wild-type and mutagenized fragments of bIAP I, III, and IV. In the process of constructing the bIAP II cDNA, several intermediate constructs, encoding functional isozymes, were produced, i.e. L1N8, INT 1, INT 2, and INT 3, that would prove useful for the identification of amino acid residues influencing the catalytic activity of these isozymes (see below). It should be noted that since the protein sequencing work identified only the residues found in the fully processed mature bIAP II molecule, the NH2-terminal signal peptide necessary for intracellular trafficking to the cytoplasmic membrane was contributed by a cDNA fragment from the bIAP IV cDNA and the COOH-terminal GPI signaling sequence was contributed by a fragment...

![Fig. 4. Mutagenesis and ligation strategy used to generate a chimeric bIAP II cDNA.](image-url)
from the bIAP I cDNA (as depicted in Fig. 4).

The kinetic characterization of the recombinant bIAP I, II, III, and IV isozymes revealed differences in their catalytic properties as shown in Table I. bIAP II and bIAP IV possess very similar $k_{cat}$ values (5900 and 6100 s$^{-1}$), about 3.4 times higher than that for bIAP I (1800 s$^{-1}$), but even bIAP III had a $k_{cat}$ value 2.4 times higher than that of bIAP I. There are considerable differences with respect to the heat stability of the isozymes. bIAP I is the most heat stable of all four isozymes displaying 13 °C higher $T_{50}$ than bIAP IV. bIAP II, and III have almost identical $T_{50}$ values, about 7 °C lower than that of bIAP I.

Site-directed Mutagenesis Identifies a D322G Exchange as Responsible for the 3.0-fold Increase of Specific Activity in bIAP II Compared with bIAP I—The more than 3-fold difference in activity between bIAP I and II, and the fact that these isozymes differ in only 24 residues (Fig. 5), provided an experimental system to attempt to uncover the residue(s) that determine the differences in $k_{cat}$ between these isozymes. Expression and characterization of the intermediate chimeric enzymes, L1N8, INT 1, INT 2, and INT 3 enabled us to rule out the role of 11 putative candidate residues. The L1N8 mutant enzyme displayed a $k_{cat}$ comparable to bIAP I, ruling out mutations at position 380, 411, 416, 420, 427, 453, and 480. Similarly the $k_{cat}$ values of the INT 3 chimeric construct was similar to that of INT 1, INT 2, and bIAP II, ruling out an effect of the N192Y substitution. To identify which of the remaining 13 residues were responsible for determining the high specific activity, the bIAP II cDNA was used as a template to mutate each position for the corresponding bIAP I residue. We tested single point mutants carrying, respectively, N122K, I133M, A142S, K180M, M205K, E310V, E326A, G329D, and I329G mutations, as well as a combined (A288Q,A294V,Q297R,L299V) bIAP II mutant (Table I). As can be seen in Table I and in Fig. 6a, the G322D mutation was, single-handedly, able to convert the kinetic properties of bIAP II into those of bIAP I. The changes included a 3-fold decrease in $K_m$, and $K_{cat}$ to values comparable to those of bIAP I (Table I). The converse mutation gave entirely consistent results, since by introducing a D322G mutation into bIAP I, the $k_{cat}$ and $K_m$ were increased in the resulting mutant to values comparable to that of bIAP II itself. Similarly, the introduction of an S322G substitution in bIAP III increased its $k_{cat}$ value to 5900 s$^{-1}$ while the S322D mutation reduced its $k_{cat}$ value to 1200 s$^{-1}$, comparable to the $k_{cat}$ values of (Asp$^{322}$)bIAP II and bIAP I. The differences in heat stability between bIAP I and bIAP II appears to be due to the combined effect of more than one substitution, since both the (Glu$^{322}$)bIAP I and the (Asp$^{322}$)bIAP II mutants display stability curves that are intermediate between those of bIAP I and II isozymes (Fig. 6b). The D322E substitution had a small destabilizing effect, i.e. about 1 °C lower than that of bIAP IV, and III have almost identical $T_{50}$ values, about 7 °C lower than that of bIAP I.
stability value of the wild-type bIAP I.

Despite the fact that no three-dimensional structure is available for any of the mammalian APs, our sequence comparisons indicate that residue 322 is located 2 amino acids away from a sequence absolutely conserved in APs throughout evolution, i.e. 311EGGRIDGHIL320, that contains three crucial ligands (Glu311, Asp316, and His320) coordinating to the active site zinc and magnesium ions. While further experimentation will be necessary to understand the detailed mechanistic effect of the D322G substitution, we can conclude from our data that the additional Asp at position 322 in bIAP I is impairing the hydrolysis of the phosphoenzyme complex during catalysis. Based on the general reaction scheme of APs (Scheme 1) (12, 20), the expression for $k_{cat}$ equals $k_{cat} = 1/(k_2 + 1/k_3)$. Hence a rise in $k_{cat}$ can occur only as a consequence of an improved enzyme phosphorylation ($k_2$) or an improved hydrolysis of the phosphoenzyme complex ($k_3$). Our kinetic data reveals that a rise in $k_{cat}$ in (Gly322)bIAP I is paralleled by a proportionally identical rise in $k_3$, resulting in a constant $k_{cat}/K_m$ ratio. Since this ratio equals $k_2/(1 + k_1/k_3)$, it follows that $k_1$ is not affected. Hence the enhanced catalytic activity in bIAP II and other Gly322-containing bIAP mutants in comparison with bIAP I results from an increase in $k_3$. In other words, Asp322 does not restrict phosphate positioning in the bIAP I active site pocket and does not impair the covalent phosphoenzyme complex formation, but it impairs the subsequent changes in coordination of the phosphate group during its hydrolysis from the active site Ser. The D322G substitution present in bIAP II relieves this interference.

Concluding Remarks—The present study has revealed an unprecedented level of complexity for the bovine IAP gene family and has shown that residues not directly participating in the mechanism of catalysis, but spatially close to the active site, are capable of influencing substrate catalysis contributing to variations in $k_{cat}$ in mammalian APs. Our findings also provide a rational explanation for the heterogeneity found in different purified commercial preparations of calf IAP. These heterogeneities result from difficulties in isolating a pure IAP isozyme from a tissue that may be expressing seven or more IAP genes. Furthermore, if more than one of the IAP genes are expressed in the same cell, heterodimers are likely to form which, as reported (20), will display noncooperative allosteric behavior where the stability and the catalytic properties of each monomer are controlled by the conformation of the second subunit. It follows that purified preparations of cow IAPs can show variations in the thermal stability, composition, and catalytic activity due to the combined effect of random heterodimer formation and multiplicity of expressed AP transcripts.

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FIG. 6. Kinetic and heat stability properties of wild-type, chimeric, and 322 mutant bIAPs. a, Hanes plots of $[pNPP]/v$ versus $[pNPP]$ for the bIAP I, bIAP II, L1N8, INT 1, (Gly322)bIAP I and (Asp322)bIAP II mutants. b, heat stability curves of the bIAP I, bIAP II, (Gly322)bIAP I, and (Asp322)bIAP II mutants.