Structural and Catalytic Similarities between Nucleotide Pyrophosphatases/Phosphodiesterases and Alkaline Phosphatases*

Received for publication, August 18, 2000, and in revised form, September 28, 2000
Published, JBC Papers in Press, October 10, 2000, DOI 10.1074/jbc.M007552200

Rik Gijsbers, Hugo Ceulemans, Willy Stalmans, and Mathieu Bollen‡
From the Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Nucleotide pyrophosphatases/phosphodiesterases (NPPs) generate nucleoside 5’-monophosphates from a variety of nucleotides and their derivatives. Here we show by data base analysis that these enzymes are conserved from eubacteria to higher eukaryotes. We also provide evidence for the existence of two additional members of the mammalian family of ecto-NPPs. Homology searches and alignment-assisted mutagenesis revealed that the catalytic core of NPPs assumes a fold similar to that of a superfamily of phospho-/sulfo-coordinating metalloenzymes comprising alkaline phosphatases, phosphoglycerate mutases, and arylsulfatases. Mutation of mouse NPP1 in some of its predicted metal-coordinating residues (D358N or H362Q) or in the catalytic site threonine (T238S) resulted in an enzyme that could still form the nucleotidylated catalytic intermediate but was hampered in the second step of catalysis. We also obtained data indicating that the ability of some mammalian NPPs to autodephosphorylate is due to an intrinsic phosphatase activity, whereby the enzyme phosphorylated on Thr-238 represents the covalent intermediate of the phosphatase reaction. The results of site-directed mutagenesis suggested that the nucleotide pyrophosphatase/phosphodiesterase and the phosphatase activities of NPPs are mediated by a single catalytic site.

Mammalian nucleotide pyrophosphatases/phosphodiesterases (NPPs) are type II transmembrane proteins with a small intracellular domain (10–80 residues) and a large extracellular domain (~830 residues) that also harbors the catalytic site. Since their catalytic site is extracellular, this family of proteins are denoted as ecto-NPPs. However, members of this family can also be processed into soluble, secreted forms by proteolysis at specific sites C-terminal to the transmembrane domain (3–5). In vitro, NPPs release nucleoside 5’-monophosphates from nucleotides and a variety of nucleotide derivatives. For example, NPPs efficiently hydrolyze ATP into AMP and PPi. This catalysis occurs via a covalent intermediate, i.e. a nucleotidylated threonine in the catalytic site (6). Some NPPs are also able to autophosphorylate and autodephosphorylate this catalytic site threonine. The autophosphorylation is associated with an inhibition of the nucleotide pyrophosphatase/phosphodiesterase activity (6).

The three known members of the mammalian ecto-NPP family have a relatively broad tissue distribution and have been implicated in a variety of cellular processes. NPP1, previously known as PC-1, is likely to be involved in soft tissue calcification and bone mineralization through its ability to generate PPi, an inhibitor of calcification and mineralization (8). However, NPP1 has also been shown to oppose insulin signaling, and this effect does not require a functional catalytic site (9). The anti-insulin effects of NPP1 may be the result of its direct interaction with the insulin receptor (10). NPP2 or autotaxin was shown to stimulate cell motility (11) and to augment the invasive and metastatic potential of ras-transformed cells (12). NPP3, also known as gp130RB13–6 (13) and B10 (5), has been reported to promote the differentiation and invasive properties of glial cells (13).

Although most of the biological effects of NPPs appear to be mediated by their NPP activity, the physiological NPP substrates remain to be identified. It is our long term goal to obtain information on the NPP substrate(s) by expressing mutants that can still form the catalytic intermediate but can no longer hydrolyze this intermediate. Characterization of this “trapped” covalent adduct should then provide data on the nature of the substrate(s). As a first step toward this goal, we report here on mutations that specifically block the second catalytic step of NPPs. These “trapping” mutants were designed on the basis of results of a structure-prediction analysis, which suggested that the catalytic domain of NPPs is structurally and catalytically related to that of well characterized enzymes such as alkaline phosphatase. Unexpectedly, these studies have also revealed that a single catalytic mechanism accounts for both the nucleotide pyrophosphatase/phosphodiesterase and the auto (de)phosphorylation activities of NPPs.

MATERIALS AND METHODS

Sequence Analysis—The organization of the genes encoding human NPP2, human NPP4, and mouse NPP5 was determined by comparing the respective cDNA sequences with the genomic sequences in the GenBank® data base, using the BLAST-2 sequences program at NCBI (14). Intron-exon boundaries were verified according to Horowitz and Krainer (15). The sequence of NPP from Candida albicans was obtained from the Stanford Sequencing and Technology Center (available via the World Wide Web). The sequences of NPPs from Caulobacter crescentus and Porphyromonas gingivalis were acquired from the Institute for
**Similarities between NPPs and Alkaline Phosphatases**

Genomic Research (available via the World Wide Web). The sequence of the *Clostridium acetylbutylicum* NPP was obtained from Genomic Therapeutics Corp. (available via the World Wide Web).

**Generation and Expression of NPP Mutants**—Various mutants of mouse NPP1 were expressed as HA-tagged fusion proteins in COS-1 cells (Figs. 6 and 8). The expression vectors were generated by a two-step procedure. First, the pSVL-SV40 vector was cut with XhoI and BamHI, and ligated with an adaptor encompassing the sequence of a HA tag (YPYDVPDYA) and a multiple cloning site, yielding the pMB001 vector. The mouse NPP1 coding sequence, obtained from the pSVL/SV40 plasmid (17), was cut with XhoI and BamHI and subcloned in the pMB001 vector. Point mutations were introduced using the QuickChange site-directed mutagenesis protocol of Stratagene. PCR amplifications were performed using *Pfu* proof-reading polymerase (Roche Diagnostics). All mutations were verified by DNA sequencing.

**Western Analysis**—Following Tricine-SDS-PAGE (7.5%) the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) by electroblotting at 38 V in 50 mM Tris-base plus 50 mM boric acid at pH 8.3. Unspecific binding sites were blocked in phosphate-buffered saline containing 5% (w/v) milk powder and 0.2% (v/v) Triton X-100. After centrifugation (5 min at 5000 g), the supernatant was washed once with 0.25M LiCl and twice with 0.5M LiCl and ligated with an adaptor encompassing the sequence of an HA tag (YPYDVPDYA) and a multiple cloning site, yielding the pMB001 vector. The mouse NPP1 coding sequence, obtained from the pSVL/SV40 plasmid (17), was cut with XhoI and BamHI and subcloned in the pMB001 vector. Point mutations were introduced using the QuickChange site-directed mutagenesis protocol of Stratagene. PCR amplifications were performed using *Pfu* proof-reading polymerase (Roche Diagnostics). All mutations were verified by DNA sequencing.

**Cos-1 cells** were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and 100 units/ml each of penicillin and streptomycin. The cells were transfected with the NPP constructs containing 10% (v/v) fetal bovine serum and 100 units/ml each of penicillin and streptomycin. The cells were transfected with the NPP constructs containing 10% (v/v) fetal bovine serum and 100 units/ml each of penicillin and streptomycin. The cells were transfected with the NPP constructs.
A Conservation of secondary structure

Panel A, the consensus secondary structure of the catalytic domain of mouse NPP1 (residues 192–568) was predicted by combination of the PSI-PRED, PHD, SamT99, JPRED, and Prof secondary-structure prediction algorithms. This predicted structure was aligned with the known secondary structure of the *E. coli* AP (PDB code 1ALK), the *B. stearothermophilus* iPGM (PDB code 1EJJ), and the human AS-A (PDB code 1AUK), AS-B (PDB code 1FSU), as determined with the Swiss-PdbViewer (41). The regular numbers indicate the distances to the beginning and the end of each protein, and the numbers in parentheses indicate the sizes of the gaps between the aligned segments. The superscript numbers show the position of some key residues in each protein. β-Strands are shown in gray boxes and are numbered, while the α-helices are in a black box and are lettered. *, Catalytic site residue; Δ, residues coordinating Zn$^{2+}$ (Zn1) in AP and Mn$^{2+}$ (Mn1) in iPGM; ▲ residues coordinating Zn$^{2+}$ (Zn2) in AP and Mn$^{2+}$ (Mn2) in iPGM, Mg$^{2+}$ in AS-A and Ca$^{2+}$ in AS-B; ◊, residue coordinating Mg$^{2+}$ in AP. Panel B shows a model of the catalytic core of NPPs, as deduced from the conserved fold elements and known structure of APs, iPGMs, and ASs. The α-helices and β-strands are numbered and lettered as in panel A. The position of the active site threonine and of the residues involved in the binding of metals (Me) are denoted as in panel A. Points in the surface loops where additional protein fragments should be inserted are indicated by breaks. For none of the aligned proteins did the used set of secondary prediction programs (see above) forecast an α-helical structure for helix B. However, since this fragment is known to adopt an α-helical structure in APs, iPGMs, and ASs, it was assumed that the corresponding fragment of NPP1 also folds into an α-helix.

B Model of the catalytic core of NPPs

![Model of the catalytic core of NPPs](image-url)
FIG. 4. Model of the catalytic core of NPPs. The primary structure of the catalytic domain of mouse NPP1 was threaded onto the known three-dimensional structure of the catalytic domain of alkaline phosphatase (PDB code 1ALK). Using the known (AP) and predicted (NPP) secondary structure as criteria (see Fig. 3A), the six Zn$^{2+}$-coordinating residues of AP showed a superposition with identical residues in all NPPs. In addition, the catalytic site Thr of NPP1 coincided with that of the catalytic site Ser of AP. The figure shows the predicted constellation of the metal-binding residues and the catalytic site Thr of mouse NPP1. A similar active site constellation has been derived from the crystal structure of AS-A, AS-B, and iPGM (30–32). Red, oxygen; blue, nitrogen; green, metal (Me).

**RESULTS AND DISCUSSION**

**A Family of NPP Proteins**—A search of the nonredundant sequence data base at NCBI with NPP1–3, using the BLAST-algorithm (21), resulted in the identification of two novel mammalian NPP homologues, designated NPP4 and NPP5 (Fig. 1). An EST analysis revealed a rather broad tissue distribution of the families of arylsulfatases (AS), phosphopentomutases, 2,3-bisphosphoglycerate-independent phosphoglycerate mutases (iPGM), and the alkaline phosphatases (AP). The latter four families have previously also been classified in a superfamily of phospho-/sulfo-carbohydrate processing metalloenzymes, based on the conservation of metal binding motifs (28). Furthermore, the crystal structures of *E. coli* AP (PDB code 1ALK), human AS-A and AS-B (PDB codes 1AUK and 1FSU, respectively) and *Bacillus stearothermophilus* iPGM (PDB code 1EJJ) have been solved (29–32), which has provided extensive proof of structural and catalytic similarities.

A PSI-BLAST search with the sequence of *E. coli* AP (SwissProt P00634) attributed a higher similarity score to NPPs than to AS (data not shown). Conversely, when the human AS-A sequence (SwissProt P15289) was used as query, NPPs emerged with a higher similarity score than did alkaline phosphatases. Thus, within the superfamily of phospho-sulfo-coordinating metalloenzymes, the NPP family is positioned intermediate between ASs and APs. Since the catalytic domains of APs, ASs, and iPGMs have been shown to assume a basically identical fold, i.e. a $\beta$-sheet sandwiched between $\alpha$-helices (29–32), it seems likely that the catalytic domain of NPPs would adopt the same fold. In agreement with this view, the fold recognition program GenTHREADER (33), which uses
a threading potential to evaluate the quality of fold assignments, proposes with the highest level of confidence that the fold of the human AS-A and AS-B occurs in the catalytic domain of human NPP1.

Since a combination of various, independent prediction methods has been shown to improve the reliability of secondary structure prediction (34, 35), a consensus secondary structure was predicted for the catalytic domain of mouse NPP1, with the use of the PSI-PRED, PHD, SamT99, JPRED, and Prof prediction algorithms (36–40). The predicted secondary structure was aligned with the known secondary structure common to the catalytic domains of APs, iPGMs, and ASs. This alignment revealed a remarkable conservation in the length and position of \( \alpha \)-helices and \( \beta \)-strands between these four families (Fig. 3). By threading the sequence of the predicted secondary structure elements of NPP1 onto the known secondary structure of the AP, iPGM, and AS backbones, we were able to produce a rough structural model of the catalytic domain of NPPs (Fig. 3B). A striking feature of this model is that the metal-binding and active site residues in NPP1 are superposed onto those of AP and iPGM. Thus, the 6 residues in APs and iPGMs that are known to coordinate Me1 and Me2 (see also Fig. 4).

The Reaction Mechanism of NPPs—Since we predicted the catalytic site of NPPs to have an arrangement similar to that of APs and iPGMs, this also suggested a similar catalytic mechanism. Transposing the known reaction mechanism of APs (44) and iPGMs (32) to NPPs yields the reaction scheme shown in Fig. 5. It is proposed that the Me2-activated nucleophilic hydroxyl of Thr-238 attacks the phosphate of the incoming substrate, resulting in the formation of a covalent, nucleotidylated intermediate. In the second catalytic step, a Me1-activated water molecule attacks this E-NMP intermediate, regenerating Thr-238 and releasing a nucleoside 5'-monophosphate. An essential difference between NPPs and APs lies in the nature of their respective substrates, i.e., a phosphodiester and a phosphomonoester, respectively. In APs the substrate affinity is increased by an arginine (Arg-166 in E. coli AP), which is hydrogen-bonded to the two oxygen atoms of the phosphate moiety that are not interacting with metal ions. In the typical NPP substrates, however, one of these oxygens is

---

Fig. 5. Proposed reaction mechanism for NPPs. The proposal is based on the known reaction mechanism of APs (44) and iPGMs (32), and the conserved catalytic site structure elements of NPPs, including the residues that coordinate Me1 and Me2 (Figs. 3 and 4). The open circles represent OH- or O-groups. \( R \) refers to various structures, including a nucleoside monophosphate (e.g., in AP2A), a phosphate (e.g., in ADP), or a pyrophosphate (e.g., in ATP). In the free enzyme (E), the hydroxyl group of the catalytic site threonine (Thr-238 in mouse NPP1) is stabilized by Me2 in its nucleophilic state. The enzyme-substrate complex (E-R-O-NMP) is formed as a result of the coordination of the NMP-ester oxygen atoms by Me1 and additional interaction of one of the nonbridging oxygen atoms of the substrate with Me2. Thr-238 (T238) holds a position opposite to the leaving group of the NMP-ester substrate. A nucleophilic attack of the hydroxyl group of Thr-238 results in the formation of the covalent, nucleotidylated intermediate (E-NMP), departure of the alcohol leaving-group and inversion of the phosphorus center. At alkaline pH a water molecule coordinated by Me1 attacks the phosphorus apically, resulting in the hydrolysis of the nucleotidylated intermediate, inversion of the phosphorus center, and the formation of a noncovalent enzyme-NMP complex (E-NMP). The free enzyme is regenerated by dissociation of NMP (nucleoside monophosphate). Also shown are the residues that coordinate Me1 and Me2 (see also Fig. 4).
Similarities between NPPs and Alkaline Phosphatases

A Western blot analysis

B Phosphodiesterase activity

C Covalent nucleotidylation

FIG. 6. Mutations affecting the NPP activity and/or the formation of the nucleotidylated intermediate of NPP1. Mouse NPP1 or the indicated mutants were expressed in COS-1 cells as fusions with an HA tag. The fusion proteins were immunoprecipitated from the cell lysates with anti-HA antibodies. Similar amounts of immunoprecipitated fusion proteins, as quantified by Western analysis with antibodies against the C terminus of NPP1 (panel A), were used for the assay of phosphodiesterase activity with p-nitrophenyl thymidine 5'-monophosphate as substrate, in the absence (light gray bars) or presence (dark gray bars) of 2 mM ZnCl₂ (panel B). The immunoprecipitated NPP1 fusions were also used for the trapping of the nucleotidylated intermediate with 50 μM α-[γ-³²P]ATP as substrate (panel C). The nucleotidylated intermediate was visualized by autoradiography after SDS-PAGE. The mutation of the catalytic site residue, i.e. T238S or T238A, decreased the NPP activity by 95 and 100%, respectively (Fig. 6B). By contrast, mutation of the neighboring residues (K237A and F239A) merely halved the activity. Mutation of any of the 6 residues (Figs. 4 and 5) predicted to be involved in the binding of Me1 (Asp-358, His-362, and His-517) or Me2 (Asp-200, Asp-405, and His-406) essentially abolished the NPP activity (Fig. 6B). Mutation of the corresponding residues in E. coli AP and B. stearothermophilus iPGM has been reported to be inhibitory as well (41, 51–57).

It can be envisaged that the activity of NPP1 that is mutated in its metal-coordinating residues, can be (partially) restored by the addition of an excess of metals. Since the identity of the metals in the catalytic site of NPPs is not known, we have first explored which metals can restore the activity of NPP1 following metal chelation by 1 mM EDTA. We found that the activity of EDTA-inhibited NPP1 could be largely restored by the mere addition of 2–5 mM amounts of either ZnCl₂ or CaCl₂, but only partially by 2–5 mM MgCl₂ (data not shown). In Fig. 6B it is shown that the activity of the H362Q and H517Q mutants of NPP1 was also restored to about 60% of the control value by the addition of 2 mM ZnCl₂. The activity of the other putative metal coordination mutants (D405N, H406Q, D358Q, D200N) was merely halved the activity. Mutation of any of the 6 residues (Asp-358, His-362, and His-517) or Me2 (Asp-200, Asp-405, and His-406) essentially abolished the NPP activity (Fig. 6B). As expected, the inactive T238A had lost the ability to form the nucleotidylated intermediate. In contrast, the poorly active T238S mutant accumulated the nucleotidylated intermediate, which indicates that the hydrolysis of the intermediate was hampered. A similar reasoning applies to the D358N and H362Q mutants. An increased labeling of the latter mutants could be expected since Asp-358 and His-362 were proposed to be involved in the binding of Me1, which is predicted to play an essential role in the second step of catalysis (Fig. 5). The inability of the H517Q mutant to form the covalent inter-
mediate may suggest that the mutation of His-517 to a glutamine, in addition to abolishing the binding of Me1, also disturbs the coordination of Me2. Indeed, the mutation of any residue predicted to be involved in the coordination of Me2 (D200N, D405N, H406Q) abolished the formation of the nucleotidylated intermediate, in agreement with the requirement of Me2 to start the catalytic cycle (Fig. 5). The F239A mutant, which displayed about half of the wild type NPP activity (Fig. 6), also showed a decreased ability to trap the catalytic intermediate (Fig. 6C). Phe-239 is conserved in all NPPs but not in APs, iPGMs, and ASs, indicating that the role of this residue is specific for NPP substrates. For example, Phe-239 could be involved in the coordination of the substrate. If so, the decreased activity and level of covalent intermediate of the F239Q mutant may be accounted for by a decreased binding of the substrate.

NPPs Also Exhibit a Phosphatase Activity—Having concluded that NPPs have a catalytic site that is similar to that of APs, we wondered whether the capability of NPPs to autophosphorylate and autodephosphorylate (see Introduction) could perhaps reflect an intrinsic phosphatase activity, in which autophosphorylated NPP represents the covalent phospho-intermediate. We have obtained initial data that support this view. First, autophosphorylation of NPP1 was also observed with [β-32P]ADP (Fig. 7), establishing that it does not represent a classical kinase reaction. Second, under identical conditions, a labeling was also observed with AP from E. coli (data not shown) and from bovine intestinal mucose (Fig. 7), showing that ATP and ADP are indeed substrates for APs. On the other hand, neither NPP1 nor AP were labeled by incubation with [32P]P4, indicating that the labeling was not due to the generation of P4 by hydrolysis of ATP or ADP.

We have also explored the effects of point mutations at or near the catalytic site on the ability of NPP1 to become autophosphorylated (Fig. 8). Mutations D358N and H362Q still allowed a weak labeling, whereas mutation of any of the other residues proposed to be involved in the binding of Me1 (His-517) and Me2 (Asp-200, Asp-405, and His-406) completely abolished the autophosphorylation in the presence of [γ-32P]ATP. This suggests strongly that the same catalytic site mediates both the nucleotide pyrophosphatase/phosphodiesterase and the phosphatase activities. Moreover, as was observed for the phosphodiesterase reaction (Fig. 6C), the T238S mutant could still autophosphorylate (Fig. 8). Although the F239A mutant showed a reduced capacity for nucleotidylation (Fig. 6C), its ability to form a phosphorylated intermediate was not affected (Fig. 8). However, if Phe-239 is involved in the coordination of the nucleoside moiety, as suggested above, it can be expected that its mutation decreases the phosphodiesterase activity without affecting the phosphatase activity since both enzymic reactions require the substrate to be bound in a different way (see also “Conclusions and Perspectives”). Unexpectedly, although the D358N and H362Q mutants showed an increased labeling with [α-32P]ATP (Fig. 6C), the labeling with [γ-32P]ATP was decreased (Fig. 8). These data suggest that Asp-358 and His-362, in addition to their role in coordinating Me1, may also play a role in the binding of nucleotides as substrates for the phosphatase reaction.

Conclusions and Perspectives—We have made use of the wealth of information in protein data bases to gain some initial insight into the catalytic mechanism of NPPs. Rather unexpectedly, this analysis has revealed that NPPs belong to the superfamily of phospho-sulfo-coordinating metalloenzymes. The detailed knowledge of the structure and catalytic mechanism of some of these enzymes has helped us to identify residues in NPPs that are required for catalysis. The essential role of these residues has subsequently been confirmed by site-directed mutagenesis. Interestingly, mutation of some of the residues that were predicted to be involved in the second catalytic step (Thr-238, Asp-358, His-362) generated enzymes that accumulated the nucleotidylated intermediate.

Our studies have also provided an entirely novel insight into the dual function of NPPs, i.e. as nucleotide pyrophosphatases/phosphodiesterases and as auto(de)phosphorylating enzymes. We suggest that the ability of some NPPs to auto(de)phosphorylate is actually a reflection of an intrinsic phosphatase activity, whereby the autophosphorylated enzyme represents the covalent intermediate of the phosphatase reaction. Based upon the relative production rates of ADP, AMP, PPi, and P4 by NPP1 (16) or NPP2 (58), it can be deduced that their phosphatase and nucleotide pyrophosphatase/phosphodiesterase activities are of similar order of magnitude. If anything, the contribution of the phosphatase activity was underestimated in these studies since ADP, a product of the phosphatase reaction, is also a substrate of the NPP reaction. It should also be noted that the ratio of both enzymic activities is bound to be substrate-dependent. Thus we noted that p-nitrophenylphosphate, a classical substrate of alkaline phosphatases, is not hydrolyzed to a measurable extent by NPP1 (data not shown).

The similarities between the catalytic core of NPPs and APs (Fig. 3), combined with the results of site-directed mutagenesis (Fig. 8), strongly suggest that the phosphatase and nucleotide pyrophosphatase/phosphodiesterase reactions are catalyzed by the same catalytic site. At first glance, phosphatases and phosphodiesterases catalyze a completely different reaction. However, it can be argued that phosphatases hydrolyze phosphate esters whereas NPPs hydrolyze nucleotidyl esters (or acid anhydrides), resulting in the removal of phosphate or nucleotidyl groups, respectively. Replacing a free hydroxyl group of the substrate-bound phosphate moiety by a nucleoside transforms a phosphatase reaction scheme into a phosphodiesterase reaction scheme. For NPPs to act both as nucleotide pyrophosphatases/phosphodiesterases and as phosphatases, one only has to imply that (some) substrates can be bound in two different ways. For example, with ADP as substrate it is either the α- or the β-phosphate that will become covalently bound to the catalytic site threonine in the pyrophosphatase or phosphatase reaction, respectively. Future investigations should provide a deeper insight into the binding modes of the substrates and into the relative importance of the nucleotide pyrophosphatase/phosphodiesterase and the phosphatase reactions that are catalyzed by NPPs.

Acknowledgments—Karolien Nelissen provided expert technical assistance. We acknowledge Monique Beulens for the preparation of NPP1 antibodies.

REFERENCES
1. Goding, J. W. (2000) J. Leukocyte Biol. 67, 285–311
2. Goding, J. W., Terkelstra, R., Maurice, M., Deterrer, P., Sali, A., and Belli, S. I. (1998) Immunol. Rev. 161, 11–26
3. Belli, S. I., Van Driel, I. R., and Goding, J. W. (1993) Eur. J. Biochem. 217, 421–428
4. Stracke, M. L., Krutzsch, H. C., Unsworth, E. J., Arestad, A., Ciocé, V., Schiffermann, E., and Liotta, L. A. (1992) J. Biol. Chem. 267, 2329–2335
