Acidic Residues at the Active Sites of CD38 and ADP-ribosyl Cyclase Determine Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) Synthesis and Hydrolysis Activities*  

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a novel metabolite of NADP that has now been established as a Ca²⁺ messenger in many cellular systems. Its synthesis is catalyzed by multifunctional enzymes, CD38 and ADP-ribosyl cyclase (cyclase). The degradation pathway for NAADP is unknown and no enzyme that can specifically hydrolyze it has yet been identified. Here we show that CD38 can, in fact, hydrolyze NAADP to ADP-ribose 2’-phosphate. This activity was low at neutrality but greatly increased at acidic pH. This novel pH dependence suggests that the hydrolysis is determined by acidic residues at the active site. X-ray crystallography of the complex of CD38 with one of its substrates, NMN, showed that the nicotinamide moiety was in close contact with Glu¹⁴⁶ at 3.27 Å and Asp¹⁵⁵ at 2.52 Å. Changing Glu¹⁴⁶ to uncharged Gly and Ala, and Asp¹⁵⁵ to Gln and Asn, by site-directed mutagenesis indeed eliminated the strong pH dependence. Changing Asp¹⁵⁵ to Glu, in contrast, preserved the dependence. The specificity of the two acidic residues was further demonstrated by changing the adjacent Asp¹⁴⁷ to Val, which had minimal effect on the pH dependence. Crystallography confirmed that Asp¹⁴⁷ was situated and directed away from the bound substrate. Synthesis of NAADP catalyzed by CD38 is known to have strong preference for acidic pH, suggesting that Glu¹⁴⁶ and Asp¹⁵⁵ are also critical determinants. This was shown to be case by mutagenesis. Likewise, using similar approaches, Glu⁸⁸ of the cyclase, which is equivalent to Glu¹⁴⁶ in CD38, was found to be responsible for controlling the pH dependence of NAADP synthesis by the cyclase. Based on these findings, a catalytic model is proposed.

The Ca²⁺ releasing activity of nicotinic acid adenine dinucleotide (NAADP)³ was first demonstrated in sea urchin eggs (1, 2) and has since been shown in a wide variety of cell types, from plants to human (reviewed in Refs. 3–5). NAADP is endogenously present in cells and its level is modulated by various agonists and stimuli, establishing its role as a second messenger (Refs. 6 and 7 and reviewed in Refs. 8 and 9). In contrast to the widespread functional activity of NAADP in cells, only two homologous proteins, mammalian CD38 and Aplysia ADP-ribosyl cyclase, have so far been identified as enzymes responsible for the synthesis of NAADP (10).

CD38 is a membrane-bound protein first thought of as a lymphocyte antigen (reviewed in Refs. 11–13) but has since been found to be widely expressed in virtually all tissues examined (14–17). It is present on the cell surface in some cases but is also localized in various intracellular organelles (15, 18–20). The Aplysia ADP-ribosyl cyclase, on the other hand, is a soluble protein that shares about 30% sequence identity with CD38 (21) and is found in large quantities in the Aplysia ovotestis (22, 23). Both CD38 and the cyclase are novel multifunctional enzymes capable of synthesizing NAADP from NADP by catalyzing the exchange of nicotinamide in NADP with nicotinic acid (10). Both can also cyclize NAD to produce cyclic ADP-ribose (cADPR) (10, 24), another novel Ca²⁺ messenger (reviewed in Refs. 3–5). The crystal structures of both proteins have been solved (25, 26) and the catalytic glutamate residues at their enzymatic active sites identified by site-directed mutagenesis (27, 28). Expectedly, a large degree of structural homology is observed between them (26).

As a second messenger, NAADP levels in cells are expected to respond to physiological stimuli. Indeed, agonists and stimuli are shown to activate rapid and transient changes in NAADP levels (7, 29, 30), indicating the presence in cells of efficient pathways for not only the synthesis but also the degradation of NAADP. As a nucleotide, NAADP is sensitive to degradative enzymes, such as nucleotide phosphodiesterase or alkaline phosphatase (31, 32). Whether these general enzymes are responsible for its degradation in cells is not known. NAADP is insensitive to regular NADase (32), and so far, no enzyme has yet been identified that can specifically hydrolyze NAADP. Here we show, unexpectedly, that CD38 is able to specifically hydrolyze NAADP to ADP-ribose 2’-phosphate (ADPRP). The

cADPR, cyclic ADP-ribose; ADPR, ADP-ribose; ADPRP, ADP-ribose 2’-phosphate; cADPRP, cyclic ADP-ribose 2’-phosphate; HPLC, high pressure liquid chromatography; WT, wild type.

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The abbreviations used are: NAADP, nicotinic acid adenine dinucleotide; HPLC, high pressure liquid chromatography; WT, wild type.

The atomic coordinates and structure factors (code 2HCT) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: NAADP, nicotinic acid adenine dinucleotide;
reaction shows a dramatic pH dependence. Using x-ray crystallography and site-directed mutagenesis, we identified two acidic residues at the active site of CD38 to be responsible for controlling both the hydrolysis and synthesis of NAADP. These results are found to be applicable for the Aplysia cyclase as well.

**EXPERIMENTAL PROCEDURES**

**Expression of the Human CD38 and Its Mutants in Yeast**—The Pichia expression vector pPICZaA (Invitrogen) was used and the construct consisted of cloning the catalytic domain of either the wild type or mutant human CD38 in frame with the yeast mating α-factor signal sequence, as described previously (27, 28, 33). The first 44 residues of CD38, containing the transmembrane and cytoplasmic domains (34), were deleted and the catalytic domain started at Arg45. The tightly controlled alcohol oxidase promoter AOX1 was used to regulate the expression of the construct. Following a 24-h growth phase in YPD (yeast extract-peptone-dextrose) medium, protein expression was sacrificed with carbon dioxide gas. The tissues were homogenized in a buffer containing 25 mm Tris–HCl, 0.1 mm EDTA, pH 7, with 15 strokes in a Potter/Elvehjem homogenizer (1:10, w/v). The homogenate was centrifuged at 15,000 rpm for 15 min. After removing the supernatant, the pellet was reconstituted in buffer and centrifuged for 15 min at 15,000 rpm. The wash procedure was repeated and the membranes were used in the assay for NAADPase activity. Brain membranes (3.5 mg/ml) were incubated with 2 mm NAADP, 50 mm sodium acetate, pH 6, for 10.5 and 26 h at 30°C. Spleen membranes (1.88 mg/ml) were incubated with 0.6 mm NAADP, 50 mm sodium acetate, pH 6, for 24 h at 30°C. The reactions were stopped by dilution (1/10) in 0.5% SDS and the products analyzed by HPLC.

**Enzyme Assays**—The NAADPase reaction was measured by incubating wild type or mutant CD38 (10 μg/ml, 1–60 min) at room temperature with 0.5 μmol of NAADP, 9 μg/ml bovine serum albumin, and at pH 4 to 8. The pH buffers used were 25 mm sodium acetate (pH 4–6) or 25 mm Tris–HCl, pH 7 or pH 8. The base-exchange reaction was assayed using 1 mm NAADP and 25 mm nicotinic acid at various pH values, adjusted with Tris base. The total volume of the reaction mixture was 100 μl, and the reaction was stopped by the addition of 150 mm HCl. The protein was removed by filtration using Immobilon-P plates (Millipore). The samples were neutralized with Tris base and diluted with water to a final volume of 1.1 ml, and 0.8 ml of the mixture was injected onto an AG MP-1 column (10 × 120 mm). The reaction products were analyzed by HPLC (BioCad). AG MP-1 was obtained from Bio-Rad. The elution was performed using a gradient of trifluoroacetic acid at a flow rate of 5 ml/min as described previously (27). Table 1 lists the maximal enzymatic activities and the pH maxima for the wild type and mutant proteins. All assays were done in quadruplicate, and the error bars indicate standard deviations.

**RESULTS**

Incubation of NAADP with CD38 resulted in progressive disappearance of the nucleotide (Fig. 1). Analyses by HPLC...
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These results suggest that the nicotinic acid moiety of NAADP is bound close to some acidic residues at the active site of CD38. At neutral pH, the electrostatic repulsion between them would reduce substrate binding and consequently the reaction rate.

X-ray crystallography was used to determine substrate binding to the active site of CD38. Although we have recently solved the crystal structure of CD38 (26), co-crystallizing CD38 with a substrate was not feasible because of its catalytic activities converting the substrate to product during the process. Instead, we first crystallized an inactive mutant of CD38, E226G, that had the catalytic glutamate residue at position 226 changed to glycine (28, 35, 36). A single E226G crystal was then soaked in a solution containing the substrate, NMN, to allow its diffusion into the active site. The structure of the complex was solved to the resolution of 1.95 Å (Table 2). Fig. 3 shows that NMN is bound inside a pocket near the middle of the protein. Detailed structural analyses of the NMN complex and complexes of other substrates will be presented elsewhere. However, what is clear from Fig. 3 is that the amide group of the NMN is in close contact with two acidic residues, 3.27 Å from Glu146 and 2.52 Å from Asp155. Assuming that NAADP binds to the active site in a similar fashion, the carboxyl group of the nicotinic acid moiety would be repelled by the two acidic residues, reducing substrate binding and reaction rate. Changing these acidic residues to uncharged ones should eliminate the electrostatic repulsion and increase the reaction rate at neutral or alkaline pH when the nicotinic acid group is charged.

Site-directed mutagenesis was used to change Glu146 to either glycine or alanine. We have previously shown that mutations of Glu146 greatly increase the NAD cyclization activity of CD38 that produces cADPR (36). Consistently, the mutants not only hydrolyzed NAADP but also cyclized it to cyclic 2'-phospho-ADP-ribose, similar to that seen with the cyclase (described later). Both reactions released nicotinic acid that was measured by HPLC and grouped together as “NAADPase” activity (Fig. 4). The pH profiles of the mutants are shown in Fig. 4 in comparison with that of the wild type (WT), which has no NAADPase activity at pH 8. The activity was restored to 80.8 ± 4.3% of the maximal activity at pH 5 (listed in Table 1) when the Glu146 was changed to glycine and to 65.1 ± 0.9% when it is changed to alanine. Similarly, changing Asp155 to Gln restored the activity to 63.9 ± 10.0% of the maximum. This effect of

| TABLE 2  
| Crystallographic data and refinement statistics  
| Values in parentheses are from the highest resolution shell.  
| 
| Data collection*  
| Cell dimensions (Å)  
| a = 41.72, b = 52.82, c = 65.59  
| Angle (°)  
| α = 106.26, β = 91.85, γ = 95.25  
| Space group  
| P1  
| Resolution (Å)  
| 30.1-1.95 (2.02-1.95)  
| Unique reflections  
| 37,216  
| Multiplicity  
| 3.8  
| I/σ  
| 18.0 (3.0)  
| Rmerge (%)  
| 6.2 (39.3)  
| Completeness (%)  
| 97.1 (95.5)  
| 
| Refinement  
| R factor (%)  
| 19.81  
| Rfree factor (%)  
| 24.76  
| Protein atoms  
| 4,006  
| Water molecules  
| 257  
| Ligands (atoms)  
| 44  
| Root mean square deviations  
| Bond lengths (Å)  
| 0.015  
| Bond angles (°)  
| 1.459  
| *R_{merge} = \sum[I - \langle I \rangle]/\sum I, where I is the integrated intensity of a given reflection; R = \sum[F_{calc} - |F_{obs}|]/\sum|F_{calc}|. R_{free} was calculated using 5% of data excluded from refinement.

![FIGURE 1. Hydrolysis of NAADP by CD38. The reaction was performed at pH 4 and 7. The products were analyzed by HPLC and the chromatographs of the reaction at pH 4 at various indicated times are shown in the inset. NA, nicotinic acid.](image1)

![FIGURE 2. The pH profiles of the NADPase and NAADPase reactions. CD38 was incubated with NADP or NAADP at the indicated pH values. The reaction products were analyzed by HPLC. The reaction rates at various pH were normalized and compared with the maximal values at pH 5.](image2)
residue change is specific since changing Asp$^{155}$ to another acidic residue, Glu, produced only minimal enhancement (12.9 ± 0.3%) at pH 8 and the overall pH profile remained essentially the same as the wild type.

Catalytically, the Aplysia cyclase is very similar to CD38, except that when NAD is used as substrate, the cyclase cyclizes it to cADPR while CD38 mainly hydrolyzes it to ADPR, with only a small amount of cADPR produced as well (22, 24, 40). It is thus of interest to determine whether the cyclase can also use NAADP as a substrate. The HPLC chromatograph in the inset of Fig. 5 shows that incubation of the cyclase with NAADP resulted in its conversion to cyclic ADP-ribose-2-phosphate (cADPRP), which eluted 2.5 min after NAADP and 1.8 min earlier than ADPRP (inset in Fig. 1). In the process, nicotinic acid was released also. The identification of cADPRP was done by treatment with alkaline phosphatase, converting it to authentic cADPR as previously described (10). Similar to the NAADPase activity of CD38, the cyclization of NAADP by the cyclase exhibited strong pH dependence and was measurable only when it was more acidic than pH 6 (Fig. 5). The value for $K_m$ was 61 ± 19 μM, and $V_{max}$ was 6,315 ± 555 nmol/mg/min, as determined by double-reciprocal plots at pH 4.

We have previously shown by crystallography that nicotinamide binds to the active site of the cyclase and is close to Glu$^{146}$ (27) that is homologous to Glu$^{146}$ in CD38. Consistent with that observed in CD38, mutating Glu$^{98}$ to glycine shifted the pH profile of the NAADP cyclization reaction toward alkaline by two pH units (Fig. 5).

The rather dramatic pH dependence of NAADP hydrolysis and cyclization is reminiscent of the synthesis of NAADP via the base-exchange reaction catalyzed by both CD38 and the cyclase (10). It is thus of interest to determine whether mutations described above have similar effects on the exchange reaction. This is the case as shown in Fig. 6. The pH profile of the base-exchange reaction catalyzed by the mutant, E146G, became essentially flat as compared with that of the wild type (upper panel, Fig. 6). Similarly, changing Glu$^{98}$ of the cyclase to glycine increased the exchange reaction at pH 6 from undetectable in the wild type to 83.4 ± 3.2% of the maximal activity (lower panel, Fig. 6).

The upper panel of Fig. 7 summarizes the results of the NAADPase activity of CD38. At pH 8, the wild type has no measurable activity. Changing Glu$^{146}$ to uncharged alanine or glycine, or Asp$^{155}$ to glutamine or asparagine, increased the activity to 50–80% of the maximal activity of the respective mutants at acidic pH (see Table 1). Changing Asp$^{155}$ to another acidic residue (Glu) produced minimal enhancement. The enhancement is specific for the two acidic residues, since changing the aspartate residue, Asp$^{147}$, immediately adjacent to Glu$^{146}$, to a valine produced only minimal activity enhancement, not more than that observed in D155E. This is consistent with the crystal structure of the CD38-NMN complex (Fig. 3). Even though Asp$^{147}$ is adjacent to Glu$^{146}$, it is directed far away from the bound NMN. It is not expected to exert significant electrostatic repulsion to the nicotinic acid moiety of NAADP. Thus changing Asp$^{147}$ to the uncharged valine had very little effect in enhancing the NAADP hydrolysis reaction.

The cyclase also can use NAADP as substrate, but instead of hydrolyzing it to ADPRP, the cyclase cyclizes it to cADPRP. The critical acidic residue in this case is Glu$^{98}$. The lower panel of Fig. 7

**FIGURE 3.** The crystal structure of CD38 complexes with NMN. Site-directed mutagenesis was used to change the catalytic residue of CD38, Glu$^{226}$ to Gly. The inactive mutant protein was crystallized, and preformed crystals were incubated with NMN to allow diffusion of the substrate into the active site. The upper panel shows the surface of the protein. The bound NMN is shown in yellow. The three relevant residues Glu$^{146}$, Asp$^{147}$, and Asp$^{155}$ are shown in space-filled models. The lower panel shows a stereo view of NMN in relation to three residues. The amide group of NMN (yellow) is 2.52 Å from Asp$^{155}$ (magenta) and 3.27 Å from Glu$^{146}$ (cyan). Asp$^{147}$ is shown in gray.
shows that the same critical acidic residues at the active site of CD38 and the cyclase just described are also important in controlling the NAADP synthesis via the base-exchange reaction.

To show that the NAADPase is physiologically relevant and can be detected in tissue extracts, mouse brain and spleen membrane fractions were incubated with NAADP and the products analyzed by HPLC. The NAADPase activities were similar in both tissues and measured to be $0.50 \pm 0.04$ nmol/mg/h for brain and $0.41 \pm 0.04$ nmol/mg/h for spleen.
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DISCUSSION

The results presented in this study are consistent with the following catalytic model. NAADP first binds to the active site of CD38 with the nicotinic acid close to Glu146 and Asp155. In the case of the cyclase, the binding is close to Glu98, which is homologous to Glu146 in CD38. The homologous residue for Asp155 is an uncharged residue, Asn107. The electrostatic repulsion between the acidic residues and the nicotinic acid moiety dictates that the substrate binding can only occur at acidic pH when either the residues or the nicotinic acid are neutralized, consistent with the fact that the NAADP reactions can occur only in acidic pH. The crystal structures of CD38 complexed with NMN shown in this study (Fig. 3) and the cyclase complexes with nicotinamide published previously (27) are consistent with this placement of NAADP in the active sites. This substrate placement is quite different from that seen in CD157, another homolog of CD38. In that case, the nicotinamide ring of NMN is mainly positioned by a Trp instead and interacts with other residues only indirectly via bound water (41). It should be noted that although CD157 is homologous to CD38, it has only a small fraction of catalytic activities of CD38. It thus appears that the close interactions of the substrate with the acidic residues seen in CD38 and the cyclase may in fact be important in positioning the substrate for the efficient action of the catalytic residues.

The next step of the reaction is the cleavage of the C1’-N1 glycosidic linkage by the catalytic action of Glu226 in CD38 or Glu179 in the cyclase, forming an intermediate. In the process the nicotinic acid ring is released. Nucleophilic attack of the intermediate by water then results in hydrolysis of NAADP and the formation of ADPRP.

A similar catalytic process can be proposed for the synthesis of NAADP via the base-exchange reaction. In this case the substrate is NADP, whose binding to the active site is not repelled by the acidic residues because the amide group of the substrate is uncharged. The lack of pH dependence of the NAADPase reaction (cf. Fig. 2) is consistent with this notion. Similar to that proposed above, catalytic release of the nicotinamide ring from the bound NADP and subsequent attack of the intermediate by water results in hydrolysis of NADP (NAADPase reaction, Fig. 2). On the other hand, if an efficient nucleophile, such as nicotinic acid, is present, its preferential attack on the intermediate will lead to the base-exchange reaction and the formation of NAADP. As shown by the cyclase-nicotinamide complex (27), nicotinic acid may well be binding close to the same critical acidic residues in the active sites during the base-exchange reaction. That changing these residues has effects essentially the same on both the exchange and the hydrolysis reactions is consistent with the scheme.

The rather unusual acidic dependence of the synthesis and hydrolysis of NAADP can have physiological relevance. It has previously been proposed that the NAADP-mediated Ca2+ signaling pathway may be specifically associated with the acidic organelles of the endocytic process (3, 42). Recent results have now shown that NAADP indeed specifically targets the lysosomal Ca2+ stores in both invertebrate and mammalian cells (43–45).

As a messenger molecule, NAADP must be removed efficiently once its signaling function is completed. Exactly how this is accomplished in cells remains to be elucidated. Based on the sensitivity of the 2’-phosphate of NAADP to phosphatases, we have proposed that the general presence of these hydrolytic enzymes in cells should ensure efficient degradation of NAADP (3). Indeed, brain membranes are able to dephosphorylate NAADP to NAAD (46). The hydrolysis reaction catalyzed by CD38 described here represents the second reaction ever shown to be able to degrade NAADP. Both reactions can be detected in brain membrane fractions with the phosphatase reaction being about 4-fold higher that the hydrolysis reaction (data not shown). The NAADPase of CD38 is, however, much more specific for NAADP and related molecules. More importantly, that CD38 is capable of both synthesizing and hydrolyzing NAADP would ensure that the degradation pathway is at the precise location of NAADP synthesis, facilitating its efficient removal. It is thus likely that the NAADPase of CD38 would play a more dominant role as a physiological removal system for NAADP.

CD38 is clearly a unique protein. Its novelty lies not on the fact that it is a multi-functional enzyme capable of using different substrates and producing distinct products but that all these products have been shown to be have novel physiological functions. CD38cyclizes NAD to produce cADPR, a second messenger for mobilizing the Ca2+ stores in the endoplasmic reticulum (reviewed in Refs. 3–5). It also hydrolyzes NAD to ADPR, which, together with cADPR, has recently been shown to be a modulator of the TRPM2 channels (47–50). At acidic pH, CD38 can produce NAADP, another Ca2+ messenger specifically targeting the lysosomal Ca2+ stores. Perhaps, most surprising of all, is the fact that CD38 is the only known enzyme that can hydrolyze cADPR to ADPR (24, 40) and, as shown in this study, NAADP to ADPRP as well, terminating the Ca2+ signaling functions of the two messengers. The base-exchange reaction catalyzed by CD38 has now been shown to be more versatile than just producing NAADP; it can in fact produce a series of adenylc dinucleotides from cADPR and adenine, which are regulators of cell proliferation (51). Finally, when CD38 is expressed on the lymphocyte surface, it also functions as an antigen and when ligated by antibodies produces a myriad of functional effects on the cells (reviewed in Refs. 11 and 52). Understanding the mechanisms and controlling factors of the multitude of functions of CD38 is clearly relevant, and by unraveling the mechanism behind the dramatic pH dependence, this study represents a step toward that goal.

REFERENCES

1. Clapper, D. L., Walseth, T. F., Dargie, P. J., and Lee, H. C. (1987) J. Biol. Chem. 262, 9561–9568
2. Lee, H. C., and Aarhus, R. (1995) J. Biol. Chem. 270, 2152–2157
3. Lee, H. C. (1997) Physiol. Rev. 77, 1133–1164
4. Lee, H. C. (2001) Annu. Rev. Pharmacol. Toxicol. 41, 317–345
5. Lee, H. C. (2002) Cyclic ADP-ribose and NAADP. Structures, Metabolism and Functions, Kluwer Academic Publishers, Dordrecht, the Netherlands
6. Billington, R. A., Ho, A., and Genazzani, A. A. (2002) J. Physiol. (Lond.) 544, 107–112
7. Yamasaki, M., Thomas, J. M., Churchill, G. C., Garnham, C., Lewis, A. M., Cancela, J.-M., Patel, S., and Galione, A. (2005) Curr. Biol. 15, 874–878
8. Lee, H. C. (2003) Curr. Biol. 13, R186–R188
