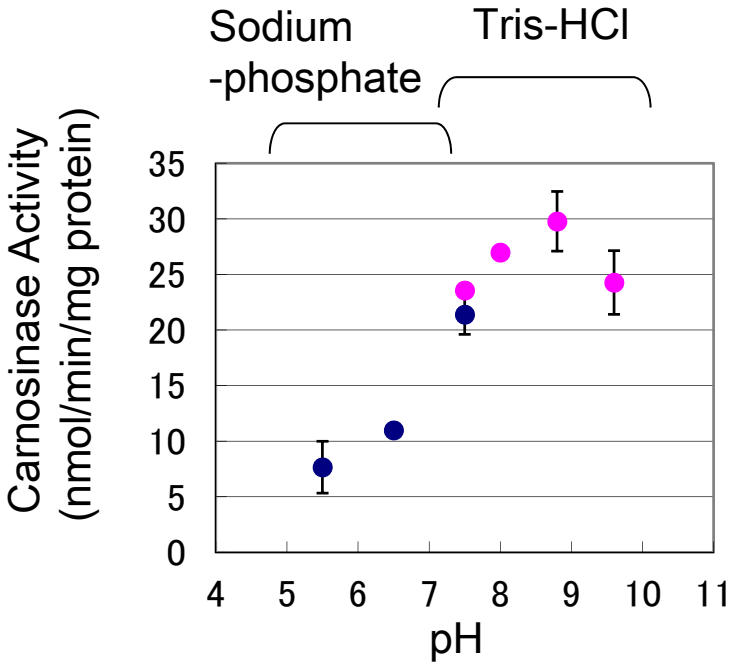
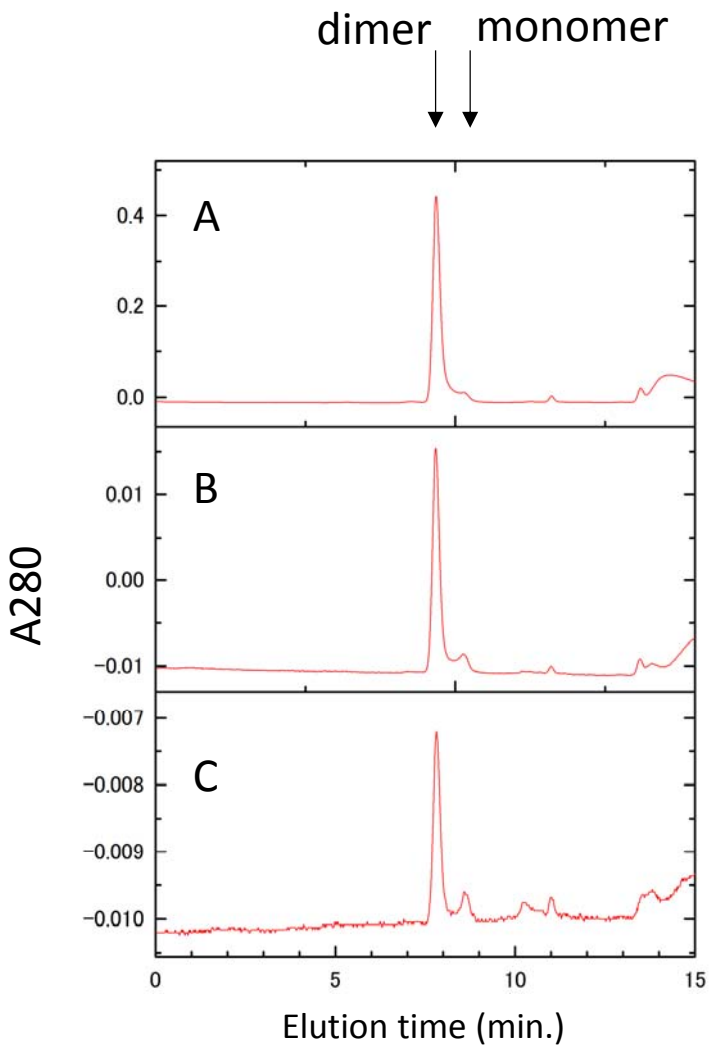


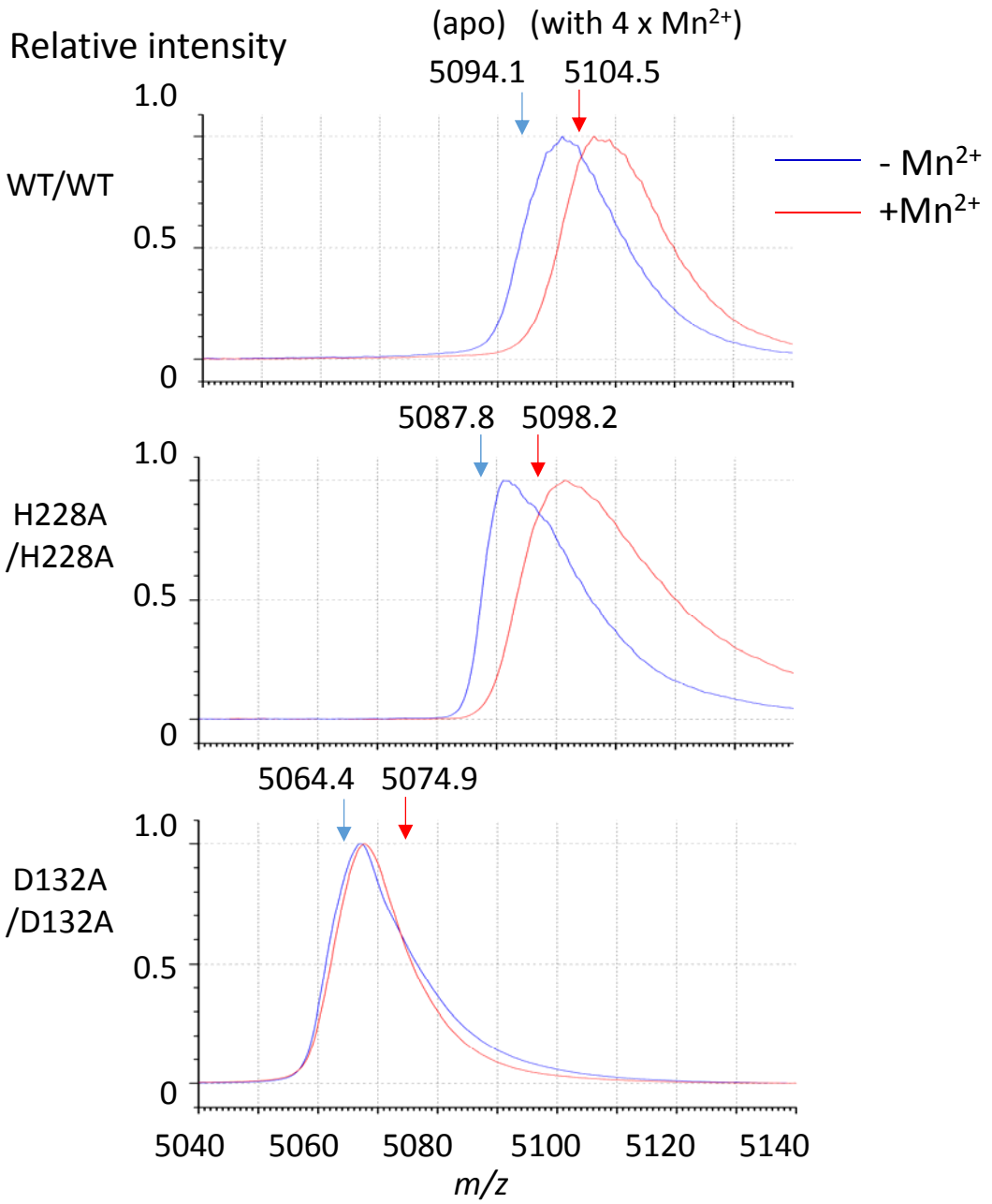
Supplementary Fig. S1.
Carnosine-hydrolyzing activity of CN2

Enzyme activity of CN2 was determined using carnosine as the substrate under different pH conditions. Buffers used were sodium phosphate (blue) or Tris-HCl (magenta) adjusted at various pH values as indicated. Data were represented as mean ± SEM of triplicated determinations.



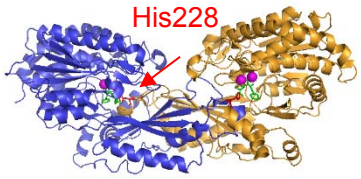
Supplementary Fig. S2.
Size exclusion chromatography of wild type CN2

Wild type CN2 was prepared at different concentrations (A: 10 µM, B: 1 µM, C: 0.1 µM) in 100 mM ammonium acetate, 1 mM DTT, pH7.5. Then 3 µl of a sample solution was applied on a size exclusion column (4.7 x 300 mm) pre-equilibrated with the same buffer, and eluted at 0.35 ml/min using an HPLC system. Expected elution positions of CN2 monomer and dimer are indicated by arrows.

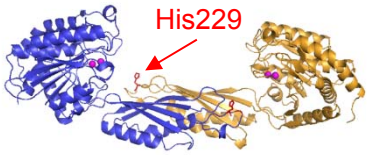


Supplementary Fig. S3.
Manganese-binding properties of H228A/H228A and D132A/D132A homodimers

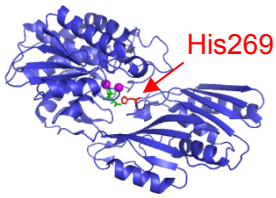
H228A/H228A or D132A/D132A homodimer prepared in 100 mM ammonium acetate, pH 7.4, was incubated with (blue) or without (red) 50 µM $MnCl_2$ for 5-15 min at room temperature and analyzed by ESI-TOF MS. The +21 charged ions were presented in this figure with the y-value of each peak apex as 1. The overall spectral patterns were basically the same as that shown in Fig. 2A. Red and blue arrows indicate the theoretical average mass values of apo-protein dimers and those with four Mn^{2+} ions, respectively.



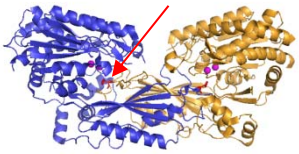
Carnosine dipeptidase II (CNDP2)
(*Mouse*, PDB : 2ZOF)



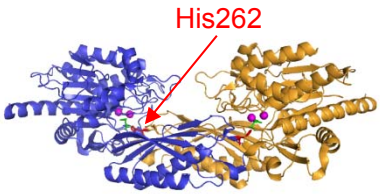
Carboxypeptidase G2
(*Pseudomonas sp.*,
PDB:CPG2)



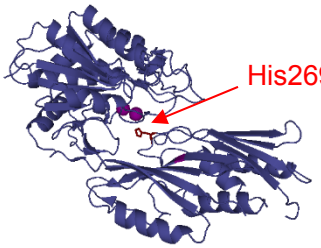
PepV (*Lactobacillus delbrueckii*,
PDB:1LFW)



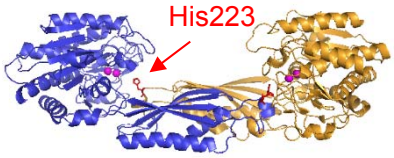
Carnosine dipeptidase I
(CNDP1)
(*Homo sapiens*, PDB : 3DLJ)



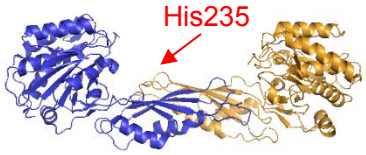
β-alanine synthase
(*Saccharomyces Kluyveri*,
PDB:2V8H)



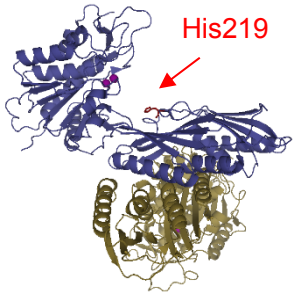
Sapep (*Staphyloconnus aureus*,
PDB:3KI9)



Tripeptide aminopeptidase,
PepT
(*E. Coli*, PDB: 1VIX)



Acetylornitine deacetylase, ArgE
(*Bacteroides thetaiotaomicron*,
PDB:3CT9)



PepD (*Vibrio alginolyticus*,
PDB:3MRU)

Supplementary Fig. S4
Comparison of crystal structures of M20 family metalloproteins.

Crystal structures of prototypes of M20 family member proteins were drawn in ribbon diagrams. The conserved His residues corresponding to H228 of CN2 were shown in red with side chains. Structural data are obtained from PDB database and the accession numbers were shown in the figure.

(A)

Charge	Observed mass (*m/z*)	Calculated mass of wild type CN2 dimer (*m/z*)
+ 24	4462.3	4457.5
+ 23	4658.2	4651.3
+ 22	4870.3	4862.3
+ 21	5100.3	5094.1
+ 20	5355.1	5348.8
0	107082 ± 23	106954.0

(B)

Charge	Observed mass (*m/z*)	Calculated mass of wild type CN2 monomer (*m/z*)
+ 16	3347.2	3343.3
+ 15	3570.2	3566.1
+ 14	3825.1	3820.8
+ 13	4119.4	4114.6
0	53538.5 ± 0.9	53477.0

Supplementary Table S1

Comparison of calculated and observed mass values of wild type CN2 monomer and homodimer in ESI-TOF MS analysis.

Wild type CN2 protein prepared using pGEX-6P3 vector was analyzed by ESI-TOF MS in aqueous (Fig. 2A) or acetonitrile-containing (Fig. 2B) solvents, and m/z values of peak apexes were presented in tables (A) and (B), respectively, together with theoretical average mass values. Mass values of whole protein dimer and monomer were calculated from the observed mass values and presented at the bottom of each table as mean ± standard deviation.

Charge	Observed (*m/z*)	Calculated (*m/z*) (I319K monomer)
+ 16	3357.4	3344.3
+ 15	3580.2	3567.2
+ 14	3835.6	3821.9
0	53691.6 ± 9.5	53492.1

Supplementary Table S2

Comparison of observed and calculated average mass values of I319K CN2 mutant in ESI-TOF MS analysis.

A CN2 mutant protein, I319K, prepared using pGEX-6P3 vector was analyzed by ESI-TOF MS (Fig. 3B), and the m/z values of peak apexes were presented together with theoretical average mass values. Mass value of whole protein monomer was then calculated from the observed mass values of +14-+16-charged ions and presented as mean ± standard deviation.

Charge	H228A /H228A		H228A/D132A		D132A/D132A	
	observed (*m/z*)	calculated (*m/z*)	observed (*m/z*)	calculated (*m/z*)	observed (*m/z*)	calculated (*m/z*)
+ 23	4646.7	4654.4	4635.5	4634.8	4624.5	4624.1
+ 22	4858.2	4856.5	4846.4	4845.4	4834.7	4834.8
+ 21	5089.2	5087.8	5076.7	5076.1	5064.4	5064.4
+ 20	5343.3	5342.1	5330.7	5329.8	5317.9	5317.6
0	106851 $\pm$ 5	106822.0	106595 $\pm$ 4	106576.7	106337 $\pm$ 5	106331.4

Supplementary Table S3

Observed and calculated mass values of H228A/H228A, H228A/D132A and D132A/D132A CN2 mutant dimers

Homodimers of CN2 mutants, H228A/H228A and D132A/D132A, were mixed, incubated at room temperature, and analyzed by ESI-TOF MS (Fig. 7A). The m/z values of peak apexes measured after 270 min incubation were presented together with theoretical average mass values. Mass values of whole protein dimers were calculated from the observed mass values of +20-+23-charged ions and presented at the bottom of the table as mean $\pm$ standard deviation.