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Increasing in Archaeal GlcNAc-1-P Uridyltransferase Activity by Targeted Mutagenesis while Retaining its Extreme Thermostability

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UDP-GlcNAc, an activated and essential form of GlcNAc which is an important component in the polysaccharide structure of most organisms, is synthesized from GlcNAc-1-P and UTP by GlcNAc-1-P UTase. We previously reported the identification of the extremely thermostable ST0452 protein, which has dual sugar-1-P NTase activities (Glc-1-P TTase and GlcNAc-1-P UTase activities) from an acidothermophilic archaeon, *Sulfolobus tokodaii* strain 7. Detailed analyses of the protein indicated that the activity is slightly lower than that of bacteria. For industrial applications, activity needs to be increased without decreasing thermostability. Therefore, to enhance this activity, we introduced mutations into the amino acid residues located within the predicted reaction centre by targeted mutagenesis. All 12 mutant ST0452 proteins showed no decrease in thermostability. Among them, six mutant proteins were found to have increased GlcNAc-1-P UTase activity under optimal reaction conditions with sufficient substrates or an appropriate metal ion. Our results indicate that targeted mutagenesis is a powerful technique for *in vitro* production of a thermostable enzyme with enhanced activity. The results of this study also indicate that the space for the metal ion is important for selecting the type of metal ion and also affects the rate of the reaction.

Key words: GlcNAc-1-phosphate uridyltransferase, improvement of enzymatic activity, targeted mutagenesis, thermostable enzyme, UDP-GlcNAc.

Abbreviations: dTDP-Glc, thymidine diphosphate glucose; Glc-1-P, glucose-1-phosphate; Glc-1-P TTase, glucose-1-phosphate thymidylyltransferase; GlcNAc-1-P, *N-acetyl*-D-glucosamine-1-phosphate; GlcNAc-1-P UTase, *N-acetyl*-D-glucosamine-1-phosphate uridyltransferase; sugar-1-P NTase, sugar-1-phosphate nucleotidylyltransferase; UDP-GlcNAc, uridine diphosphate *N-acetyl*-D-glucosamine.

In most organisms, many different types of modified sugar molecules are required to maintain biological processes such as energy production, construction of cell surface structures and synthesis of DNA and RNA molecules. In most microorganisms, many important modified sugars are synthesized from simple sugar molecules that are taken up from the surrounding environment.

N-Acetyl-D-glucosamine-1-phosphate (GlcNAc-1-P) is synthesized by a four-step reaction from glucose-6-phosphate, which is catalysed by phosphoglucose isomerase, glutamine:fructose-6-phosphate amidotransferase, phosphoglucosamine mutase and glucosamine-1-phosphate acetyltransferase. GlcNAc-1-P, the final product of this biosynthetic pathway, is converted to its activated form, uridine diphosphate *N*-acetyl-D-glucosamine (UDP-GlcNAc), by combination with UTP. This molecule is important for constructing many biological polymers. In bacteria, UDP-GlcNAc is required for synthesis of lipopolysaccharides, peptidoglycan, enterobacterial common antigen, and teichoic acid (*1–3*). In archaea, the GlcNAc moiety is a major component of the cell surface structure (*4*, *5*). In eukarya, the activated molecule is essential for the synthesis of chitin, a major component of the fungal cell wall (*6*), and the glycosylphosphatidylinositol linker, a molecule anchoring a variety of cell surface proteins to the plasma membrane (*7*). The GlcNAc moiety is found in the polycarbohydrate structure *N*- or *O*-linked to the proteins as a post-translational modification (*8–11*). As glycosylation is the most important modification for activating peptide drugs, UDP-GlcNAc, an activated form of the GlcNAc molecule, is thought to be important for future development of effective drugs.

Enzymes with *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase (GlcNAc-1-P UTase) activity, which catalysed the reaction combining GlcNAc-1-P with UTP, were identified and characterized from several bacteria, which were mainly pathogens (*12–14*). However, these enzymes are not suitable for use in application because of their low stability and possible contamination with pathogenic molecules. A stable enzyme with GlcNAc-1-P UTase activity is useful for the *in vitro* production of UDP-GlcNAc and artificial production of UDP-GlcNAc analogues labelled with isotope tracer molecules or dyes used to identify the mechanism and function of GlcNAc in polysaccharide structures.

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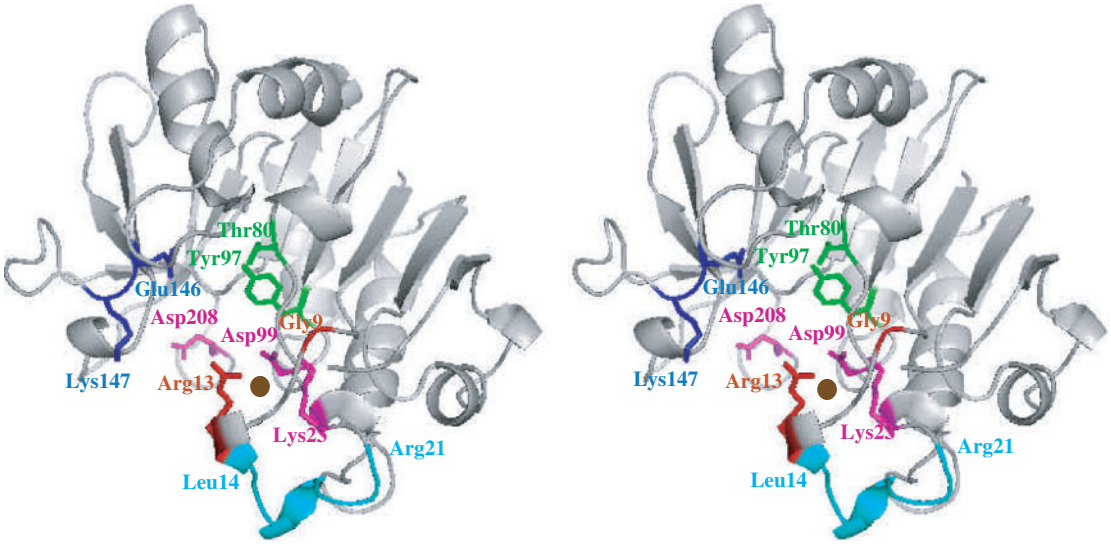

Fig. 1. **Proposed 3D structure of the domain containing the sugar-1-P NTase reaction centre of the ST0452 protein.** The stereo view of the 3D structure of the domain from 1st to 208th residues of the ST0452 protein constructed by the 3D-JIGSAW program (version 2.0) is shown. The amino acid residues participating in binding with nucleoside triphosphate substrates, sugar-1-P substrates, *N*-acetyl portion of GlcNAc-1-P and metal ions are indicated by red, blue, green and magenta, respectively. The region from Leu14 to Arg21 indicating high conservation with the corresponding sequences of *E. coli* RmlA is showing by cyan. The metal ion is indicated by brown.

In our previous study, the ST0452 protein was identified as an extremely thermostable dual-functional sugar-1-phosphate nucleotidylyltransferase (sugar-1-P NTase), glucose-1-phosphate thymidylyltransferase (Glc-1-P TTase) and GlcNAc-1-P UTase activities, from an acidothermophilic archaeon, *Sulfolobus tokodaii* strain 7 (*15*). Due to the high stability and the safety of the host microorganism, this enzyme is potentially useful in industrial applications.

In the present study, to increase the GlcNAc-1-P UTase activity of the ST0452 protein further without decreasing its thermostability, specific amino acid substitutions were introduced at the predicted reaction centre (Fig. 1). Although the *Km* values for substrates of all 12 mutant proteins constructed in this study were markedly increased compared with wild-type ST0452 protein, six modified proteins showed higher GlcNAc-1-P UTase activity under optimal reaction conditions. The information for the mechanism of the GlcNAc-1-P UTase reaction catalysed by the ST0452 protein was also obtained from detailed functional analyses of some mutant ST0452 proteins. To our knowledge, this is the first report demonstrating improvement of an archaeal enzyme with sugar-1-P NTase activity and thermostability.

EXPERIMENTAL PROCEDURES

Materials—UTP, dTTP, GlcNAc-1-P, glucose-1-phosphate (Glc-1-P), UDP-GlcNAc and dTDP-glucose (dTDP-Glc) were obtained from Sigma Chemical Co. (St Louis, MO, USA). The restriction enzymes and T4 DNA ligase used in this work were purchased from New England BioLabs, Inc. (Beverly, MA, USA). The KOD-plus DNA polymerase used for PCR amplification was obtained from Toyobo Co., Ltd (Osaka, Japan). The plasmid vector pET21(b) was purchased from Novagen (Madison, WI, USA). *Sulfolobus tokodaii* strain 7 (JCM10545) was obtained from Japan Collection of Microorganisms (JCM). *Escherichia coli* strain DH5α was purchased from Takara Bio Inc. (Otsu, Shiga, Japan) for plasmid cloning and the strain BL21-Codon Plus (DE3)-RIL was obtained from Stratagene (La Jolla, CA, USA) for expression of recombinant protein.

Construction of Expression Vectors for the Mutant ST0452 Protein—All mutations used in this work on the ST0452 coding region were introduced into PCR amplified fragments with synthetic primers possessing the site-directed conversion of nucleotide. The fragments with mutations replaced the corresponding region of the parental ST0452H plasmid, which was constructed in the previous work (*15*). The primers listed in Table 1 and primers P1 and P3 constructed in the previous work (*15*) were used to amplify the ST0452 coding region with mutations.

To convert the amino acid residues of Gly9, Lys23 and Tyr97 to Ala, Tyr97 to Phe and the residues Leu14-Glu-Pro-Ile-Thr-His-Thr-Arg21 (LEPITHTR) to Met-Tyr-Ser-Asp-Leu (MYSDL) (the region shown by the horizontal line in Fig. 2), the PCR fragments were amplified with primers MP01, MP02, MP03, MP04 or MP05 and primer P3. After digestion of these fragments with the restriction enzymes listed in the right column of Table 2, the digested fragments were purified by agarose gel electrophoresis. Each purified fragment was ligated with the pST0452H DNA digested with the same restriction enzymes. After confirmation by sequencing, the constructed plasmids with a

Table 1. **Primer designed.**

Primer ID	Sequences	Restriction enzyme
MP01	ATAGCATATGAAGGCATTTATTCTTGCTGCA**gcg**TCTGGTG	NdeI
MP02	ACAAGGCCT**gcg**GCTTTTGTTCCAATTCTC	StuI
MP03	AGGTGCGGCCATATTGTCTGCAAAGTTTAACGATGAAGCACTTATAATT**gcc**GGAGATTTA	EaeI
MP04	AGGTGCGGCCATATTGTCTGCAAAGTTTAACGATGAAGCACTTATAATT**ttt**GGAGATTT	EaeI
MP05	CTTGCTGCAGGTTCTGGTGAAAGA**atgtattccgatcttccg**AAAGCTTTTGTTCC	PstI
MP06	TTAGGCCTTGTATGCGTTATTGGTTCTAA**cgc**TTCACCAGAACC	StuI
MP07	AATATGGCCGCACC**cgc**CCCTTTTATATC	EaeI
MP08	AATATGGCCGCACC**cag**CCCTTTTATATC	EaeI
MP09	AAGAACAG**cgc**TCCATAAATTATAAG	HhaI
MP10	TGGA**gcg**CTGTTCTTTTCAAACGAGAAAG	HhaI
MP11	TATCTCGGGTTT**cgc**TATAATTTTAG	AvaI
MP12	TAAAATTATA**gcg**AAACCCGAGATACC	AvaI
MP13	GTATCTCTGG**cgc**TTCTATAATTTTAG	HhaI
MP14	CTAAAATTATAGAA**gcg**CCAGAGATAC	HhaI
MP15	TCCAAT**ggc**CATCCAATATCC	BalI
MP16	TGGATG**gcc**ATTGGAAAACCTTG	BalI

The lower cap letters indicate the nucleotides converted for introduction of amino acid conversion.
The horizontal lines indicates the recognition site of the restriction enzyme shown in the right column.
Boldface indicates the nucleotide sequences introduced for mutation.

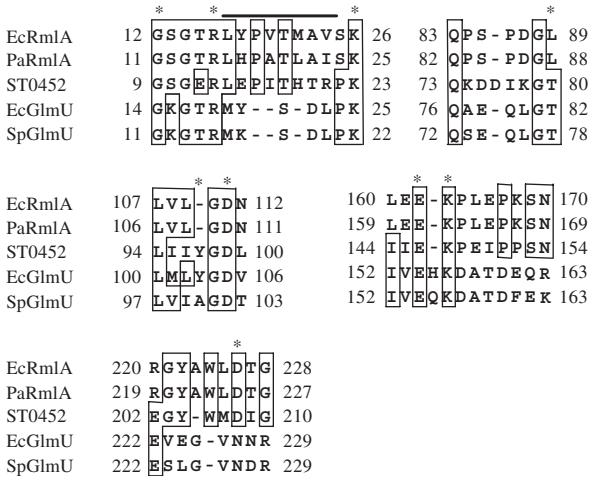

Fig. 2. **Sequence alignment of the five highly conserved domains among the ST0452 protein and bacterial Glc-1-P TTase and GlcNAc-1-P UTase.** EcRmlA and PaRmlA indicate the Glc-1-P TTase from *E. coli* (GenBank accession number P37744) and *P. aeruginosa* (NP_253850), EcGlmU and SpGlmU indicate the GlcNAc-1-P UTase from *E. coli* (YP_67180) and *S. pneumoniae* (AAK75107). The letters within boxes indicate the residues conserved over any three proteins. The amino acid residues chosen for the construction of the mutant proteins are indicated by asterisks, and the horizontal line indicates the region selected for construction of RegionI mutant protein. The numerals indicate the coordinates of the two ends of each domain from the N-terminus of each protein.

histidine tag at the C-terminus were designated as pST0452(G9A)H, pST0452(K23A)H, pST0452(Y97A)H, pST0452(Y97F)H and pST0452(RegionI)H, respectively.

To convert the amino acid residues of Arg13 and Thr80 to Ala, and Thr80 to Leu, the PCR fragments were amplified with primer P1 and primer MP06, MP07 or MP08, respectively. After digestion of these fragments with the restriction enzymes listed in the right column of Table 2, the digested fragments were purified by agarose gel electrophoresis. Each purified fragment was ligated with the pST0452H DNA digested with the same restriction enzymes. After confirmation by sequencing, the constructed vector plasmids with a histidine tag at the C-terminus were designated as pST0452(R13A)H, pST0452(T80A)H and pST0452(T80L)H, respectively.

To convert the amino acid residues of Asp99, Glu146, Lys147 and Asp208 to Ala, two PCR fragments for the 5′ and 3′ regions of the ST0452 ORF were amplified with the overlapped primers with mutation and primer P1 or P3 as indicated in Table 2. Both of the PCR fragments were digested with two restriction enzymes as shown in Table 2. These two fragments were ligated with pET21(b) DNA, which was digested with NdeI and XhoI. After confirmation by sequencing, the constructed vector plasmids with a histidine tag at the C-terminus were designated as pST0452(D99A)H, pST0452(E146A)H, pST0452(K147A)H and pST0452(D208A)H, respectively.

Expression and Purification of Recombinant Protein—The constructed plasmid expression vectors were introduced into cells of *E. coli* strain BL21-Codon Plus (DE3)-RIL. The expression and thermostability of the recombinant ST0452 proteins were monitored mainly according to the methods described in the previous report (*15*). After treatment at 80°C for 20 min, the recombinant proteins with a histidine tag were purified with a nickel-loaded HiTrap Chelating HP column (GE Healthcare Bio-Sciences Corp., NJ, USA) according to the manufacturer's protocol. Purified proteins were stored at 4°C for further analyses. The concentration of the purified protein was determined using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA)

The expressed recombinant mutant ST0452 proteins from plasmids pST0452(G9A)H, pST0452 (R13A)H, pST0452(RegionI)H, pST0452(K23A)H, pST0452(T80A)H, pST0452(T80L)H, pST0452(Y97A)H, pST0452(Y97F)H, pST0452(D99A)H, pST0452(E146A)H, pST0452(K147A)H and pST0452(D208A)H were designated as G9A, R13A, RegionI, K23A, T80A, T80L, Y97A, Y97F, D99A, E146A, K147A and D208A, respectively.

Table 2. **Combination of primers used for mutation.**

5′ region			3′ region			Plasmid constructed
Primer		Restriction enzymes	Primer		Restriction enzymes	
			MP01	P3	NdeI/XhoI	pST0452(G9A)H
			MP02	P3	StuI/XhoI	pST0452(K23A)H
			MP03	P3	EaeI/XhoI	pST0452(Y97A)H
			MP04	P3	EaeI/XhoI	pST0452(Y97F)H
			MP05	P3	PstI/XhoI	pST0452(RegionI)H
P1	MP06	NdeI/StuI				pST0452(R13A)H
P1	MP07	NdeI/EaeI				pST0452(T80A)H
P1	MP08	NdeI/EaeI				pST0452(T80L)H
P1	MP09	NdeI/HhaI	MP10	P3	HhaI/XhoI	pST0452(D99A)H
P1	MP11	NdeI/AvaI	MP12	P3	AvaI/XhoI	pST0452(E146A)H
P1	MP13	NdeI/HhaI	MP14	P3	HhaI/XhoI	pST0452(K147A)H
P1	MP15	NdeI/BalI	MP16	P3	BalI/XhoI	pST0452(D208A)H

Primers P1 and P3 were originally generated in the previouse work (*15*).

Table 3. **Kinetic properties for the GlcNAc-1-P UTase activity of the ST0452 protein.**

Protein	Substrate	*Km* (mM)	*kcat* (S^{-1})	*kcat*/*Km* (μM^{-1} S^{-1})
	Forward reaction			
ST0452	UTP	0.0017 ± 0.00035	3.53 ± 0.24	2.12 ± 0.51
	GlcNAc-1-P	0.008 ± 0.00083	2.54 ± 0.22	0.32 ± 0.043
EcGlmU	UTP	0.017	24	1.4
	GlcNAc-1-P	0.018	21	1.2
	Reverse reaction			
ST0452	UDP-GlcNAc	0.016 ± 0.0015	8.40 ± 0.59	0.53 ± 0.034
	Pyrophosphate	0.016 ± 0.0016	6.21 ± 0.45	0.38 ± 0.061

The activity of the ST0452 protein was measured at 80°C. EcGlmU indicates the GlcNAc-1-P UTase activity from *E. coli*, and the kinetic parameters for EcGlmU are according to the result described in the ref. (*16*).

Enzyme Assays—The forward reactions of GlcNAc-1-P UTase or Glc-1-P TTase activities (producing the NDP-sugar) were analysed in 10 μl of the standard reaction solution containing 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.05 μg of purified recombinant protein, and 5 μM UTP plus 50 μM GlcNAc-1-P or 100 μM dTTP plus 5 mM Glc-1-P, respectively. After 2 min pre-incubation at 80°C, the reaction was started by adding recombinant protein and progressed for 2 min and 5 min at 80°C for GlcNAc-1-P UTase and Glc-1-P TTase activities, respectively. The reaction was stopped by addition of 100 μl of 500 mM KH_2PO_4 and 50 μl aliquot was analysed on a LaChorm Elite HPLC system (Hitachi High-Technologies Corporation, Tokyo, Japan) (*15*). The reverse reaction of GlcNAc-1-P UTase was performed in the same reaction solution as the forward reaction except for replacement of substrates of UTP and GlcNAc-1-P by UDP-GlcNAc and pyrophosphate.

To determine the effect of metal ion on the GlcNAc-1-P UTase activity of wild-type ST0452 and D208A proteins, reactions were performed in the reaction solution containing 100 μM UTP, 1 mM GlcNAc-1-P, 0.1 mM EDTA and 2 mM of each metal ion.

RESULTS

Our previous report showed that the ST0452 recombinant protein, which was identified from an acidothermophilic archaeon, *S. tokodaii* strain 7, possesses both Glc-1-P TTase and GlcNAc-1-P UTase activities (*15*). As this protein was extremely thermostable, its GlcNAc-1-P UTase activity was identified as the first thermostable GlcNAc-1-P UTase activity from archaea. This enzymatic activity may be used for *in vitro* production of UDP-GlcNAc, which is an important material for construction of carbohydrate polymer structures. Therefore, a detailed analysis of the GlcNAc-1-P UTase activity of the ST0452 protein was performed.

GlcNAc-1-P UTase Activity of the ST0452 Protein—To analyse the property of GlcNAc-1-P UTase activity of the ST0452 recombinant protein, the kinetic constants for forward (production of UDP-GlcNAc) and reverse (production of UTP and GlcNAc-1-P) reactions of enzymatic activity were determined. The apparent kinetic constant values are shown in Table 3.

The previously characterized bacterial GlcNAc-1-P UTase enzymes can utilize UTP as the sole donor substrate. However, the GlcNAc-1-P UTase activity of the ST0452 protein was found to utilize both dTTP and UTP molecules. This feature was also different from the substrate specificity of its Glc-1-P TTase activity, which was capable of accepting the four deoxy-nucleoside triphosphates and UTP (*15*).

The results of analysis for the metal ions requirements for the GlcNAc-1-P UTase activity of the ST0452 protein are shown in Table 4. An absolute requirement for a

Table 4. **The enzymatic activities of the ST0452 protein in the presence of different metal ions.**

Metal ion	GlcNAc-1-P UTase activity		Glc-1-P TTase activity	
	Specific activity (μmol/min/mg protein)	Relative activity (%)	Specific activity (μmol/min/mg protein)	Relative activity (%)
Mg^{2+}	3.30 ± 0.046	100	1.32 ± 0.07	40
Zn^{2+}	1.73 ± 0.074	52	0.95 ± 0.03	29
Ca^{2+}	0.81 ± 0.019	25	ND	ND
Co^{2+}	0.65 ± 0.042	20	3.21 ± 0.17	97
Mn^{2+}	0.59 ± 0.012	19	2.17 ± 0.07	66
None	ND	ND	ND	ND

The metal ion concentration used was 2 mM. The relative activity is shown as percentage of the activity detected for GlcNAc-1-P UTase activity in the presence of Mg^{2+}. ND indicates non-detectable. All experiments were repeated four times.

divalent cation was demonstrated; no activity was detectable when a divalent cation was removed from the reaction buffer. GlcNAc-1-P UTase activity was comprehensively analysed in the presence of 2 mM of different metal ions. As shown in Table 4, the order of effectiveness of metal ions on GlcNAc-1-P UTase activity was $Mg^{2+} > Zn^{2+} > Ca^{2+} > Co^{2+} > Mn^{2+}$, which is different from that of its Glc-1-P TTase activity, $Co^{2+} > Mn^{2+} > Mg^{2+} > Zn^{2+} > Ca^{2+}$. These features will be useful for selective use of the activity.

As shown in Table 3, comparison of the *Km* and *kcat* values of the GlcNAc-1-P UTase activity of the ST0452 protein with that of the bacterial GlmU enzyme showed that the thermostable ST0452 protein was very similar to or slightly lower than GlcNAc-1-P UTase activity in bacteria (*16*). Therefore, to gain the most value from the extreme thermostability of the GlcNAc-1-P UTase activity, it is necessary to improve the activity of the ST0452 protein.

Expression of Mutant ST0452 Proteins—Random mutation is a useful technique for improving protein function, but this method requires a considerable amount of work to select the clones with the desired characteristics, and such mutations are often associated with a reduction in thermostability. Since the ST0452 protein is extremely thermostable with a half-life of 180 min at 80°C, our aim was to produce a protein with increased activity without a reduction in thermostability. To avoid reducing the thermostability of the ST0452 protein, mutations that change the overall structure of the protein were excluded. In most enzymes, the reaction centre is enclosed within the pocket structure of the protein, so it was expected that a mutation in the amino acid residues located within the reaction centre would not change the overall structure and the thermostability, and that mutation of these residues would convert the activity. To increase the GlcNAc-1-P UTase activity of the ST0452 protein, the targeted mutation was therefore introduced into the amino acid residues located within the reaction centre of the protein.

The amino acid residues to be converted were selected by comparing the sequence of the ST0452 protein with that of GlcNAc-1-P UTase from *E. coli* and *Streptococcus pneumoniae* and with that of Glc-1-P TTase from *E. coli* and *Pseudomonus aeruginosa*, the three-dimensional (3D) structures of which have already been determined (*12, 17–22*). Since the 3D structure of the ST0452 protein was not determined yet, the structure was constructed according to those of the related enzymes. The predicted 3D structure of the domain containing the sugar-1-P NTase reaction centre of the ST0452 protein is shown in Fig. 1. The amino acid residues that were predicted to play important roles in the sugar-1-P NTase activity from the complex structures of the related enzymes (*17–22*) are highlighted by colour in Fig. 1. These amino acid residues were selected as targets for introducing mutations to enhance the GlcNAc-1-P UTase activity of the ST0452 protein.

The five highly conserved regions in bacterial sugar-1-P NTase and the ST0452 protein are shown in Fig. 2. Within these regions, we chose to investigate the following amino acid residues as potential candidates for the enhancement of the GlcNAc-1-P UTase activity of the ST0452 protein.

The Gly9 and Arg13 residues (shown by red in Fig. 1) were predicted to interact with the nucleoside triphosphates (*18*). Glu146 and Lys147 residues (shown by blue in Fig. 1) were predicted to interact with the sugar-1-P substrates (*17, 20–22*). As these amino acids were charged or polar, they were converted to the Ala residue, the smallest hydrophobic amino acid. Meanwhile, the Thr80 residue (shown by green in Fig. 1) in the ST0452 protein, which was conserved among GlcNAc-1-P UTase enzymes but converted to Leu in Glc-1-P TTase enzymes, was predicted to be an important residue for recognition of the GlcNAc moiety (*20*). Therefore, the polar amino acid Thr80 was converted to Leu, a non-polar amino acid, as well as Ala. The polar amino acid Tyr97 (shown by green in Fig. 1), which was predicted to be an important residue for recognition of the *N*-acetyl portion of GlcNAc-1-P (*22*), was converted to Ala and Phe, the non-polar amino acid most similar to Tyr. As the region from Leu14 to Arg21 (shown by cyan in Fig. 1) of the ST0452 protein was detected as specific to the Glc-1-P TTase enzymes, the region was changed to Met-Tyr-Ser-Asp-Leu, the sequence of the corresponding region of the GlcNAc-1-P UTase enzyme from *E. coli*. The conversion of the region to the GlcNAc-1-P UTase type sequence was expected to enhance the GlcNAc-1-P UTase activity of the ST0452 protein. As the Lys23, Asp99 and Asp208 residues (shown by magenta in Fig. 1), which are charged amino

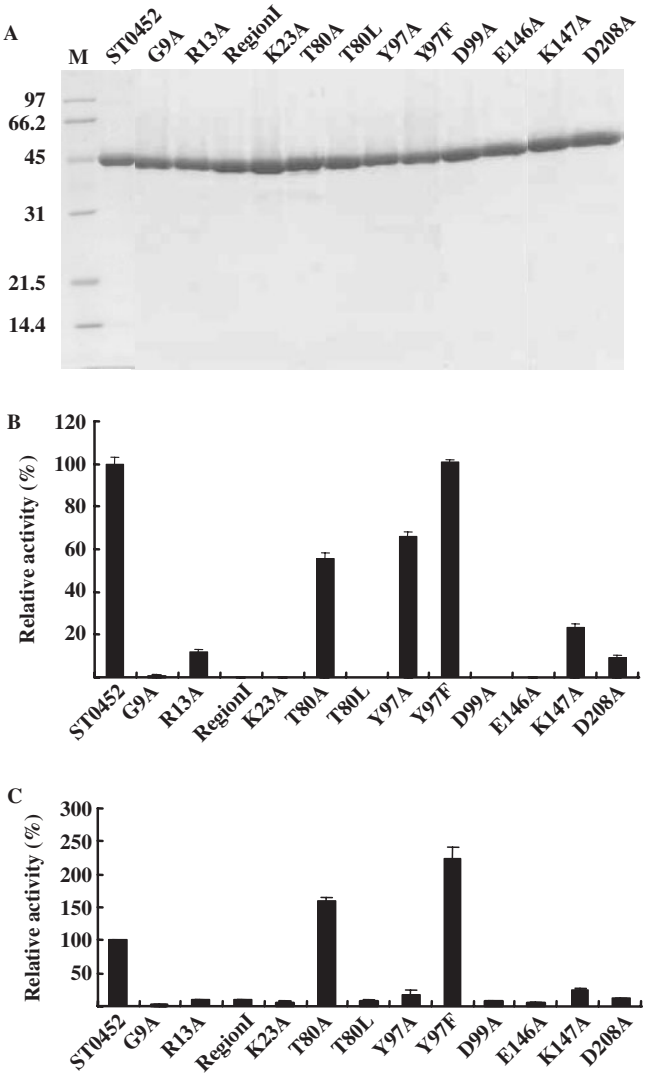

Fig. 3. **Analyses of mutant ST0452 proteins.** The recombinant mutant proteins, which were expressed in *E. coli* harbouring each mutant expression vector and purified by nickel-affinity column chromatography, analysed on 12% polyacrylamide gel containing 0.1% SDS (A). Each lane indicates the purified protein for the corresponding mutant ST0452 protein. Lane ST0452: ST0452 protein; Lane M: standard molecular mass proteins. The proteins were visualized by Coomassie brilliant blue R-250 staining. Relative activities of GlcNAc-1-P UTase (B) and Glc-1-P TTase (C) of each mutant protein. Both activities were measured with the standard reaction solution containing 5 μM UTP plus 50 μM GlcNAc-1-P, and 0.1 mM dTTP plus 5 mM Glc-1-P, respectively. The relative activities are indicated as a percentage of the activity detected on wild-type ST0452 protein. All experiments were repeated four times.

acids, were predicted to be key residues for interaction with the metal ion (*19*), these residues were converted to Ala.

All expression plasmid vectors encoding the mutant ST0452 ORFs were constructed as described in 'Experimental Procedures'. Fortunately, all mutant proteins expressed in *E. coli* were soluble after treatment at 80°C for 90 min (data not shown). The reduction in GlcNAc-1-P UTase activity on the mutant proteins after treatment at 80°C was similar to that of the parental ST0452 protein (data not shown). From these results, it can be concluded that the thermostability of all mutant proteins was not drastically changed by conversion of the targeted amino acid residues. The purity of the proteins prepared by nickel affinity chromatography after heating was monitored by SDS–PAGE. As shown in Fig. 3A, the purified proteins were detected as a single band. These proteins, therefore, were used in the following analyses.

Screening of Improvement of the Mutant ST0452 Protein Activities—To screen the conversion activity of each mutant ST0452 protein, the forward reactions of both the GlcNAc-1-P UTase and Glc-1-P TTase activities were first measured at 80°C in each standard reaction solution. As shown in Fig. 3B, only the Y97F protein showed similar GlcNAc-1-P UTase activity to wild-type ST0452 protein, and the T80A and Y97A proteins showed 56% and 66% of the activity of wild-type ST0452 protein, respectively. Conversely, for Glc-1-P TTase activity, the T80A and Y97F proteins showed higher activities than that of wild-type ST0452 protein (Fig. 3C). Although all other mutant ST0452 proteins showed little or no Glc-1-P TTase and GlcNAc-1-P UTase activity under this condition, detailed analyses of the kinetic constants for all mutants were performed to determine the effect of the various amino acid substitutions.

The result for analyses of the kinetic constants indicated that the *Km* values for sugar-1-P and nucleotide substrates of all mutant ST0452 proteins were higher than that of wild-type ST0452 protein (data not shown). Regarding the Glc-1-P TTase activity, further analysis of Glc-1-P TTase activity was not performed because of the high *Km* values for both Glc-1-P and dTTP substrates (data not shown). For the GlcNAc-1-P UTase activity, five mutant ST0452 proteins, G9A, T80A, Y97A, Y97F and K147A, and another three mutant proteins, R13A, E146A and D208A, exhibited higher and slightly reduced *kcat* values for GlcNAc-1-P UTase activity than that of wild-type ST0452 protein, respectively (data not shown). These results indicate that the GlcNAc-1-P UTase activity of the mutant ST0452 proteins were improved by the amino acid substitution. Therefore, it was expected that, after the optimization of the reaction conditions, these mutant ST0452 proteins would demonstrate increased GlcNAc-1-P UTase activity.

Optimization of the Improved Mutant ST0452 Proteins Activities—According to the above results, the GlcNAc-1-P UTase activities of the mutant proteins and the parental ST0452 protein were investigated in the presence of high concentrations of UTP substrate. GlcNAc-1-P UTase activity in the reaction solution with 100 μM UTP and 50 μM GlcNAc-1-P was monitored. As shown by the hatched bars in Fig. 4, the activity of the Y97A and Y97F mutant proteins was very similar to that of the parental ST0452 protein, and the activity of the T80A mutant protein exhibited about 30% of wild-type ST0452 protein activity under this condition. This result indicates that the GlcNAc-1-P UTase activities of the mutant proteins were not optimal. Consequently, the effect of the higher concentrations of GlcNAc-1-P

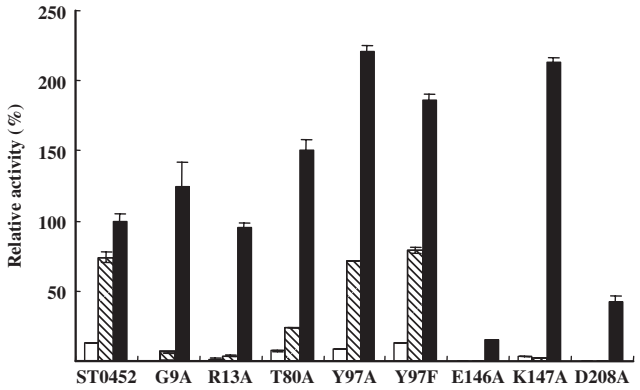

Fig. 4. **GlcNAc-1-P UTase activity of the mutant ST0452 proteins under three different reaction conditions.** GlcNAc-1-P UTase activities of each mutant protein were measured in the standard reaction solution with 5 μM UTP plus 50 μM GlcNAc-1-P (open bars), 100 μM UTP plus 50 μM GlcNAc-1-P (hatched bars) and 100 μM UTP plus 10 mM GlcNAc-1-P (black bars). The relative activity is expressed as a percentage of the activity detected on wild-type ST0452 protein under the condition containing 100 μM UTP plus 10 mM GlcNAc-1-P. All experiments were repeated four times.

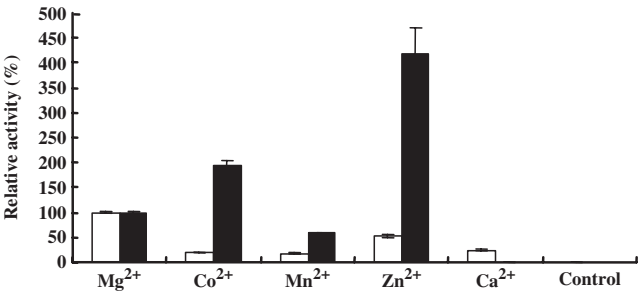

Fig. 5. **GlcNAc-1-P UTase activity of the D208A mutant protein in the presence of different metal ions.** The GlcNAc-1-P UTase activities for wild-type ST0452 protein (open bars) and D208A (black bars) were measured in the reaction solution with 100 μM UTP, 1 mM GlcNAc-1-P, 0.1 mM EDTA and 2 mM of the corresponding metal ion. The relative activity is expressed as a percentage of the activity detected on wild-type ST0452 protein when Mg^{2+} was used. All experiments were repeated four times.

substrate on the activity of each mutant protein was investigated.

GlcNAc-1-P UTase activity was measured in the reaction solution with 100 μM UTP and 10 mM GlcNAc-1-P. As shown by the black bars in Fig. 4, five mutant proteins, G9A, T80A, Y97A, Y97F and K147A, showed enhancement of GlcNAc-1-P UTase activity under this condition, revealing that the mutant proteins with high *kcat* values (data not shown) had high activity under the condition with 100 μM UTP and 10 mM GlcNAc-1-P substrate. Even under the optimal condition identified in this study, the R13A protein showed only the same GlcNAc-1-P UTase activity as wild-type ST0452 protein. Two mutant proteins, E146A and D208A, exhibited slightly weaker activity even under this condition.

As D208A was the mutant protein in which the amino acid residue probably contributing to the interaction with the metal ions (*19*) had been substituted, the effect of different metal ions on GlcNAc-1-P UTase activity was analysed. In these analyses, the reaction solutions with 100 μM UTP, 1 mM GlcNAc-1-P and 2 mM of different types of metal cation were used. As shown by the black bars in Fig. 5, when Zn^{2+} was added to the reaction solution, the activity detected was approximately four times higher than that of wild-type ST0452 protein. The addition of Co^{2+} approximately doubled the activity. These results indicate that the GlcNAc-1-P UTase activity of the D208A mutant protein is enhanced in the presence of Zn^{2+} or Co^{2+}. GlcNAc-1-P UTase activity of the D208A mutant protein was not detected in the reaction solution in the presence of Ca^{2+}.

The present work indicates that the amino acid substitution within the reaction centre improves enzymatic activity without drastically changing thermostability. Improvement in the GlcNAc-1-P UTase activity was detected in 6 of the 12 mutant proteins constructed.

DISCUSSION

GlcNAc-1-P UTase, which catalyses the synthesis of UDP-GlcNAc from GlcNAc-1-P and UTP, is essential in most organisms. The 'sequential-ordered bi bi' mechanism is currently suggested as the reaction of the related enzyme (*17*), instead of the previously proposed 'ping-pong bi bi' mechanism (*23*). This enzyme produces UDP-GlcNAc, an activated form of GlcNAc and an important precursor for many types of carbohydrate polymers such as *N*- and *O*-linked oligosaccharides as constituents of glycoproteins and glycolipids. These structures on the cell surface are essential for maintaining the integrity of the organism. The bacterial cell surface structures peptidoglycan and lipopolysaccharides are radically different from the phospholipids of the eukaryotic cell membrane. Therefore, enzymes that catalyse the reactions for synthesizing the bacterial cell surface structure are targets for the development of novel antibiotic drugs. In addition, the UDP-GlcNAc is important for *in vitro* synthesis of the polymer structure of carbohydrate molecules. Despite the importance of GlcNAc-1-P UTase activity as a model for the development of drugs and as an *in vitro* producer of UDP-GlcNAc, previous characterization of the enzyme has been performed on only a limited number of prokaryotic organisms including *E. coli* (*13, 20, 22, 24, 25*), *S. pneumoniae* (*12*, *21*) and *Neisseria gonorrhoeae* (*14*). Therefore, the identification of GlcNAc-1-P UTase with extreme thermostability was required.

In our previous work, we reported the presence of the extremely thermostable ST0452 protein, which has dual-functional sugar-1-P NTase activities, Glc-1-P TTase and GlcNAc-1-P UTase activities, and was identified from an acidothermophilic archaeon; *S. tokodaii* strain 7 (*15*). Although the ST0452 protein was originally predicted to be a Glc-1-P TTase based on its high similarity to the bacterial enzyme, detailed analyses of the enzymatic activity of the ST0452 protein conducted in the present

study indicated that the protein possesses GlcNAc-1-P UTase activity with a distinctive property, the multiple usages of the nucleotide substrates and metal ions. The current study is the first report of a detailed characterization of an extremely thermostable enzyme with GlcNAc-1-P UTase activity from archaea.

Our results provide an opportunity for application of the protein to the *in vitro* construction of a sugar nucleotide molecule, UDP-GlcNAc. However, the GlcNAc-1-P UTase activity of the ST0452 protein was slightly weaker than that of the bacterial GlcNAc-1-P UTase. Therefore, we plan to increase the GlcNAc-1-P UTase activity of the ST0452 protein to broaden the scope of its potential applications.

In previous attempts using the random mutagenesis method, it was necessary to identify a small number of mutant enzymes with enhanced activity from a large pool of constructed mutant clones (*26*, *27*). By the random method, a great deal of work was needed to select the improved proteins. The ST0452 protein exhibited the high stability with a half-life of 180 min at 80°C and 60 min at 95°C (*15*). Since thermostability is desirable for a variety of applications, we attempted to increase the GlcNAc-1-P UTase activity of the ST0452 protein without decreasing its thermostability.

The thermostability of the ST0452 protein had to be maintained throughout the mutagenesis; therefore, the amino acid residues located within the pocket of the reaction centre were selected as target residues for introducing amino acid substitutions. The hypothesis was that substituting amino acid residues inside the protein, in an area resembling a reaction pocket, would have a minimal influence on its overall structure and thermostability. As shown in Fig. 2, we selected as targets nine amino acid residues and one region which were predicted to interact with substrates within a reaction centre based on known structural information of related proteins (*12, 17–22*).

Fortunately, all of the 12 mutant ST0452 proteins were soluble and exhibited sugar-1-P NTase activity after treatment for 90 min at 80°C. None of the mutant proteins exhibited a significant difference in stability at high temperature compared with parental ST0452 protein. This observation confirmed the hypothesis that the amino acid residues located within the reaction centre were unlikely to play an important role in the 3D structure of the protein.

Among the 12 mutant ST0452 proteins constructed, six mutant proteins (G9A, T80A, Y97A, Y97F, K147A and D208A) showed increased GlcNAc-1-P UTase activity under optimal reaction conditions, in which a sufficient amount of substrate, and appropriate metal ions in the case of D208A, were supplied (Figs. 4 and 5). This increase in activity occurred despite elevated *Km* values for substrates (data not shown). The increase activity of the six mutant ST0452 proteins was thought to be due to increased in the *kcat* values (data not shown). The high success rate of the targeted mutagenesis (50%) confirmed the usefulness of this method for introducing amino acid substitutions into thermostable proteins without abolishing their thermostability and constructing proteins with enhanced enzymatic activity. These improved proteins should be more suitable for the intended applications.

In addition to the higher GlcNAc-1-P UTase activity of the ST0452 protein, introducing mutations into the ST0452 protein provided valuable information on the mechanism of its enzymatic reaction. The K23A and D99A mutant proteins had much lower *kcat* values for their GlcNAc-1-P UTase activity (data not shown) and undetectable Glc-1-P TTase activity. From the 3D structural data of related enzymes, Lys23 and Asp99 were predicted to interact with the metal ion in the reaction centre (*19*, *21*). Thus, it appears that the metal ions and the amino acid residues interacting directly with metal ions have the biggest impact on progression of the GlcNAc-1-P UTase reaction.

Table 4 shows that GlcNAc-1-P UTase activity of the parental ST0452 protein was most efficiently enhanced by Mg^{2+} ion, but that Glc-1-P TTase activity was most efficiently enhanced by Co^{2+} ion. In the previous 3D structures for Glc-1-P TTase from *P. aeruginosa* (*17*) and GlcNAc-1-P UTase from *E. coli* (*22*) and *S. pneumoniae* (*21*), three amino acid residues (Lys23, Asp99 and Asp208; shown by the coordinate in the ST0452 protein), appeared to interact directly with the metal ion. These three amino acid residues were also conserved in the ST0452 protein, but the most efficient metal ions for each activity differed between these two activities of the ST0452 protein. Mg^{2+} (atomic radius of 1.6 Å) was the most effective and Zn^{2+} (atomic radius: 1.33 Å) was the second most effective for GlcNAc-1-P UTase activity of the ST0452 protein. Conversely, Co^{2+} was the most effective and Mn^{2+} was the second most effective for Glc-1-P TTase activity of the ST0452 protein. The atomic radii of Mg^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+} and Mn^{2+} are 1.6, 1.33, 1.97, 1.25 and 1.12 Å, respectively. This correlation indicates that the ability of each metal ion to influence the activity of the ST0452 protein is related to the atomic radius of the metal ions (Fig. 6). Also, it was predicted

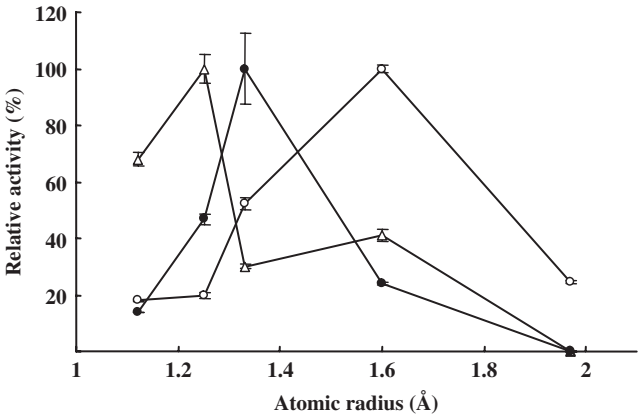

Fig. 6. **Correlation between the atomic radius of metal ions and sugar-1-P NTase activities of the ST0452 and D208A proteins.** The relative activities of the Glc-1-P TTase (open triangles) and GlcNAc-1-P UTase (open circles) activities on wild-type ST0452 protein and GlcNAc-1-P UTase (black circles) activity on D208A mutant protein in the presence of different metal ions are shown according to the atomic radius. Relative activity is expressed as a percentage of the maximum activity when the most effective metal ions were used. All experiments were repeated four times.

that the space for incorporation of the metal ions into the active centre of the protein was varied with choice of sugar-1-P and NTP substrates. As shown in Fig. 6, the difference in the atomic radius of each metal ion from that of the most efficient metal ion coincides with the order of the effectiveness of metal ions on the activities of the ST0452 protein. These results indicate that the size of the pocket for the metal ion in the reaction centre varies with the sugar-1-P and NTP substrate, and the most appropriate size of this pocket differed between GlcNAc-1-P UTase and Glc-1-P TTase activities.

The results for the D208A mutant protein indicate that the most effective metal ion for GlcNAc-1-P UTase activity changed to Zn^{2+}, with an atomic radius of 1.33 Å (Fig. 5). The same metal ion was found to be the most effective for Glc-1-P TTase activity of the D208A mutant protein (data not shown). Conversely, enzymatic activity was not detected when Ca^{2+} was added as the cofactor. This observation indicates that the space in the D208A reaction centre was altered to accommodate molecules with an atomic radius of ~1.33 Å such as Zn^{2+} (Fig. 6). Since the atomic radius of Ca^{2+} is 1.97 Å, it was thought that this metal ion was not able to enter into this space. Therefore, Ca^{2+} was not capable of increasing either the GlcNAc-1-P UTase or the Glc-1-P TTase activities of the D208A mutant protein. This suggests that D208A enzymatic activity can be regulated by adding Zn^{2+} or Ca^{2+} into the reaction solution.

The improved GlcNAc-1-P UTase with high thermostability is likely to be useful in the production of effective drugs. In addition, the strategy developed and discussed earlier is an efficient method for improving the thermostable enzymes isolated from thermophilic archaea.

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