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De Novo Design and Synthesis of Four α-Helix Bundle Proteins with Flavin Function

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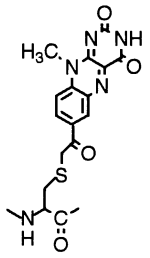
Four α-helix bundle proteins containing flavin moieties (7-acetyl-10-methylisoalloxazine) were *de novo* designed and synthesized. The artificial proteins could effectively catalyze the oxidation of dihydronicotinamides under controlled conditions for bundle structure.

The application of the amphiphilic α-helix motif[1] has been generally established in the *de novo* design of artificial proteins with a four α-helix bundle structure.[2-4] Since the inner space of the bundle structure is hydrophobic, it seems to be useful as the enzymatic active site by introducing any functional group. Recently, the enzyme-mimicking artificial proteins have been demonstrated as helichrome[3] and chymohelizyme.[4] However, the functional groups of them are not placed in the hydrophobic inside but at the ends of α-helical segments. In the present study, we attempted utilization of the hydrophobic space formed within a four α-helix bundle structure of a 53-peptide (Fig. 1) by employing a flavin moiety as a catalytic group for the demonstration of an enzyme-mimicry based on a designed polypeptide.

DAPELLKA**X**AELLK-
DAPELLKALAELLK-
DAPELLKA**Y**AELLK-
DAPELLKALAELLKG
53-peptide

53-peptide	X	Y
1	Leu	Cys(Flavin)
2	Cys(Flavin)	Cys(Flavin)



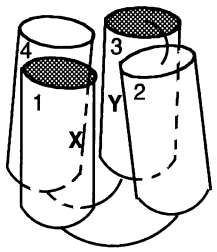


Fig. 1. Structure of flavin-containing 53-peptides and illustration of the four α-helix bundle structure. A; Ala, DA; D-Ala, E; Glu, K; Lys, L; Leu, P; Pro.

The design of artificial proteins was carried out according to the previous reports for the four-α-helix-bundled 53-peptides.[5,6] The 11-peptide was designed to form an amphiphilic α-helix structure with 3 turns of α-helix. Cysteine residue(s) were used in the third α-helix segment (Y residue in Fig. 1) or the first and the third segments (X and Y residues) to introduce one or two isoalloxazine moieties. The sequence D-Ala-Pro was

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added at the *N*-terminus of each helical segment to provide a turn property between two segments. The four 13-peptides were connected in series to a *C*-terminal Gly residue to give the 53-peptide.

Synthesis of the 53-peptides was carried out by the convergent method with solid-phase synthesis using *p*-nitrobenzophenone oxime resin and solution segment condensation.[5-7] After deprotection with HF, 7-bromoacetyl-10-methylisoalloxazine (5 equiv.)[8-10] was reacted with the side chain(s) of Cys in 0.1 M (1 M = mol dm^{-3}) Tris HCl, pH 7.5/dimethylsulfoxide (1/1, v/v) at room temperature for 5 h to give the flavo-53-peptides **1** and **2**. Peptides were identified by amino acid analysis based on Gly. Gel-permeation chromatography confirmed that 53-peptides were monomeric form. UV absorption: λ (0.1 M Tris HCl, pH 7.5)/nm; 430 (ε / dm^3 mol^{-1} cm^{-1} 11000), 342 (7700) and 284 (34600) for **1**; 430 (21800), 341 (12200) and 286 (63400) for **2**. The flavin moiety incorporated in the 53-peptides showed 1/5 less intensity in fluorescence at 510 nm (excited at 430 nm) than 7-acetyl-10-methylisoalloxazine (AcFl) as a result of thioether linkage.[8]

The 53-peptides showed highly α-helical CD pattern (60% α-helicity)[11] in 0.1 M Tris HCl, pH 7.5. With such an α-helicity in buffer solution, it has been shown that the 53-peptides take a four α-helix bundle structure by fluorescent probe methods.[5,6] The four segments are expected to form hydrophobic inner space, which may accommodate flavin moieties (X and Y residues in Fig. 1). The hydrophobic space of **1** or **2** might also effective to bind the substrates, e.g. 1-alkyl-1,4-dihydronicotinamides.

The oxidative activities of the flavo-53-peptides were examined by the oxidation of dihydronicotinamides [1-benzyl-1,4-dihydronicotinamide (BzlNAH) and 1-hexyl-1,4-dihydronicotinamide (HexNAH)] according to Levine and Kaiser.[8] The 53-peptide with a flavin **1** could oxidize both dihydronicotinamides with the rate constants k (M^{-1} s^{-1}) of 290 and 690 (Table 1). The rate accelerations of the oxidation reaction by introducing the flavin moiety to the bundle peptide were 1.35 and 0.75 times as compared to the rates with AcFl. It is noteworthy that the Michaelis-Menten parameters (Km values) for the reactions of **1** with BzlNAH and HexNAH are 3.7 and 0.15 mM, respectively. This significant difference may reflect the tightness of the binding site, which effectively distinguishes the slim n-hexyl group from the bulky benzyl one. The tight binding to HexNAH might reduce the rate of releasing the product and result in the poorer acceleration ratio (0.75) than that of BzlNAH (1.35).

Table 1. Second order rate constants for the oxidation of dihydronicotinamides by flavo-53-peptides

Flavin	k (M^{-1} s^{-1}) [a] for BzlNAH		k (M^{-1} s^{-1}) [a] for HexNAH	
	Buffer	SDS (2.0 mM)	Buffer	SDS (1.5 mM)
1	290 (1.35)	920 (3.68)	690 (0.75)	3050 (3.67)
2	660 (3.07)	1480 (5.92)	1370 (1.49)	4730 (5.70)
AcFl	215 (1)	250 (1)	920 (1)	830 (1)

a) Determined by initial rates measured by the decrease in absorbance of dihydronicotinamides at 360 nm in air-saturated 0.1 M Tris HCl , pH 7.5 containing 2% MeOH at 25 °C as reported method.[8-10] [BzlNAH] or [HexNAH] = 2.5 x 10^{-5} - 2.0 x 10^{-4} M; [Peptide] or [AcFl] = 8 x 10^{-7} M ; [Catalase] = 0.1 mg/ml. Ratios of the rate constants as compared to AcFl are indicated in the parentheses.

Therefore, on the consideration of tightness of the bundle structure in aqueous solution, we tried to loosen it by the addition of a surfactant, sodium dodecyl sulfate (SDS). Interestingly, as shown in Fig. 2, the oxidative activities of **1** to BzlNAH and HexNAH increased to 920 and 3050 as the second order rate constants at 2.0 and 1.5 mM SDS, respectively (under critical micelle concentration of SDS), while those of AcFl did not change so much (Table 1). The acceleration ratios to both substrates as compared to AcFl were 3.7. SDS at this concentration area may be effective to start melting the bundle structure.[12] The loosened (or lubricated) bundle structure might perform smoother behaviors in taking in the substrates and putting out the products. The higher concentration of SDS may completely melt the bundle structure, resulting in the diminished activities. The 53-peptide containing two flavins **2** catalyzed the oxidation more efficiently than **1** in spite of the absence or presence of SDS (for instance, in the presence of SDS, 5.9 and 5.7 for BzlNAH and HexNAH, respectively). About twice greater rate accelerations could be attributed to the doubled concentration of the flavin moieties in the hydrophobic space.

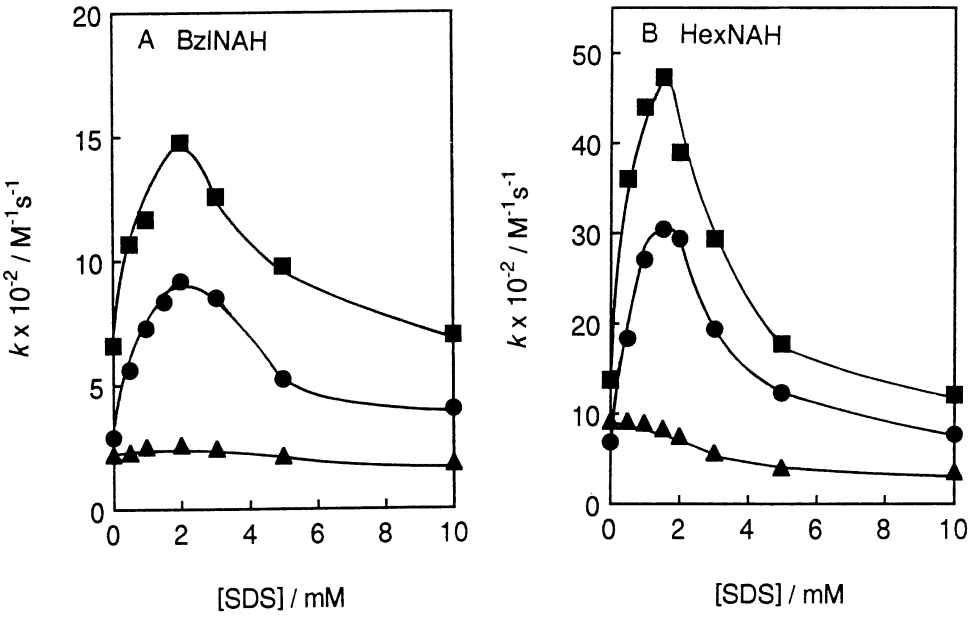

Fig. 2. Effects of SDS on the oxidation of dihydronicotinamides by flavo-53-peptides.
A; Oxidation of BzlNAH, B; Oxidation of HexNAH. (●) **1**, (■) **2**, and (▲) AcFl.

Since the behaviors of the *de novo* designed protein under various conditions are not well known yet[5,6] and presumed to be rather complicated particularly in the catalytic performance, we also examined the lubricating effect of another nonionic surfactant, polyoxyethylene(10) octylphenyl ether (triton X-100). Again, the reaction rates of **1** and **2** increased in similar profiles to those in Fig. 2 (data not shown) with maxima at 10 mg/ml of triton X-100 for BzlNAH (2.0 and 4.1 times greater than that of AcFl, respectively) and for HexNAH (1.6 and 3.2 times, respectively). Though these acceleration ratios are slightly poorer than those in the presence of SDS, the nonionic surfactant could also increase the activity of flavin moiety on the α-helical segment of polypeptide. These facts suggest that the insertion of the lipophilic tails into the inner space of the bundle structure loosens the tight bundle of α-helices to increase the mobility of the functional group. On the other hand, hydrophilic moieties of surfactants seem to play minor roles in the catalytic reaction.

Kaiser *et al.* have reported various flavo-proteins as semisynthetic enzymes.[8-10] The most successful flavo-papain showed the highest acceleration ratio (28 times for BzlNAH).[8] However, flavo-lysozyme[9] and flavo-glyceraldehyde-3-phosphate dehydrogenase[10] exhibited only a few times increase in rate accelerations (1.2 times for 1-methyl-1,4-dihydronicotinamide and 2.7 times for BzlNAH, respectively). As artificial functional proteins, our flavo-53-peptides (**1** and **2**) can be considered as successful and also promising examples in further model studies.

The flavin function was successfully provided, for the first time, in a four α-helix bundle protein by the *de novo* design method. Though the activity was not high by using only the hydrophobic inside of the amphiphilic α-helix bundle structure, it could be increased by controlling the tightness with the surfactant. It will be further increased by designing the appropriate binding pocket with multi-recognition sites.

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