The site-selective incorporation of a NAD^+ cofactor mimic into a folded helix–loop–helix polypeptide motif

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LA-42, a polypeptide with 42 amino acid residues, has been designed to fold into a hairpin helix–loop–helix motif that can dimerise in solution to form a four-helix bundle. On the surface of the folded motif a reactive site has been introduced that contains a histidine and a lysine residue in a helical sequence. The reaction between the His-Lys site and a *p*-nitrophenyl ester has previously been shown to lead to the site-selective formation of an amide at the side chain of the flanking lysine residue. An *N*-methylnicotinoyl group has now been incorporated into LA-42, in a reaction between the peptide and the *N*-methylnicotinic acid ester in aqueous solution at pH 5.9, to form a template for the engineering of selective catalysts for the reduction of carbonyl compounds. The formation of an amide bond between LA-42 and the cofactor mimic was established by electro-spray mass spectrometry and NMR spectroscopy and the reduction of the *N*-methylnicotinoyl residue by sodium dithionite in aqueous solution at pH 6.5–7 was demonstrated by UV and NMR spectroscopy. Key problems with NAD^+/NADH models in aqueous solution include poor solubility of the ox and/or red forms of the catalysts and short lifetimes of the red form due to hydrolysis of the enamine. Both the red and the ox forms of the peptide-linked nicotinoyl cofactor are soluble in aqueous solution, which is a necessary condition for the development of turnover systems, and the lifetime of the reduced form of the polypeptide catalyst has been increased by more than a factor of three over that of the 1-methylnicotinamide.

Nicotinamide adenine dinucleotide, NAD^+, is the cofactor of the alcohol dehydrogenases and NADH is the source of hydride ions in the reduction of carbonyl compounds. The mimicking of the activity of NADH, the reduced form of NAD, has been attempted in model systems but remains a formidable challenge. Key problems include stereospecificity in the hydride transfer reaction, substrate recognition, turnover and catalyst solubility. Attachment of the cofactor to a macromolecular template such as a folded polypeptide or a protein presents, in principle, possible solutions to all of those problems. Stereospecific hydride transfer and substrate recognition may be accomplished by the introduction of binding residues in the reactive site of the catalyst. Turnover and catalyst solubility in aqueous solution may be obtained since the reducing agent sodium dithionite can be added in large excess and the polypeptide template is likely to ensure solubility of both the reduced and oxidised forms of the cofactor. The engineering of cofactor-linked polypeptides is therefore of great interest and the recently reported site-selective functionalisation[1–3] reaction provides an efficient and simple route for the incorporation of the cofactor model into the folded protein.

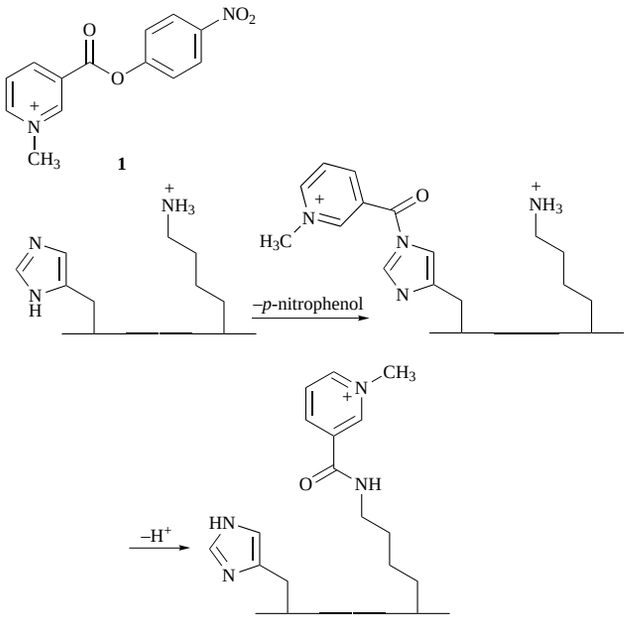
The postsynthetic incorporation of new functionalities into folded polypeptides and proteins have important applications in glycosylations, immobilisations, the engineering of novel peptide structures and the incorporation of cofactors. So far the most common approaches have been to incorporate cystein residues into the amino acid sequence[4] to carry out nucleophilic reactions or form disulfide linkages, or to react the protein with active ester derivatives. In the former method, the sensitivity of thioethers and disulfides to reducing or oxidising agents limit their range of applications and in the latter the incorporation is non selective. Yet another alternative, the incorporation of functionalised amino acids in the sequence during synthesis, requires that enantiomerically pure and side-chain protected amino acids of some complexity will have to be synthesised[5] and incorporated into the peptide. Due to peptide synthesis problems the use of amino acid conjugates is, in practice, limited to peptides with less than 20 residues.

Recently, we reported that a histidine in position i of the amino acid sequence flanked by a lysine or an ornithine in position $i + 4$ or $i - 3$ in a helical conformation reacts with *p*-nitrophenyl esters in a two-step reaction to form the corresponding amide at the side chain of the flanking residue.[1–3] The first step was found to be the formation of an acyl intermediate under the release of *p*-nitrophenol and the reactivity of the histidine towards the ester was enhanced by the flanking positively charged ammonium ion.[2] In the second step the acyl group was transferred to the amine of the flanking residue in a fast intramolecular reaction. The overall amidation reaction is faster than the corresponding bimolecular reaction between an ester and a primary amine by a factor of 10^3 which makes it highly selective. The discovered reaction provides an efficient way of incorporating new functionalities directly into the folded peptide in a single step in aqueous solution at room temperature and pH 5.9. Here, we wish to report that we have applied this reaction to the incorporation of a NAD^+ cofactor mimic into a folded helix–loop–helix motif by reacting LA-42, a polypeptide with 42 residues that folds into a helix–loop–helix motif, with *p*-nitrophenyl *N*-methylnicotinate **1**. The resulting cofactor linked helix–loop–helix motif has been designed to reduce carbonyl groups *via* hydride transfer from the reduced form of the nicotinoyl residue and it has been designed to function as a template for the incorporation of residues capable of substrate recognition and transition state binding. The development of cofactor mimic linked folded polypeptides is expected to have great potential in the study of structure–reactivity relationships in model catalysts and in the exploitation of cofactor chemistry.

Results and discussion

The design of LA-42

The amino acid sequence of LA-42, Fig. 1, is identical to that of RA-42, a polypeptide with 42 amino acid residues, except that ornithine-15 (Orn-15) of RA-42 has been replaced by a lysine (Lys-15). The solution structure of LA-42 is assumed to



Scheme 1 Reaction mechanism for the reaction between LA-42 and **1**

be the same as that of RA-42 since the only difference between them is that the side chain of Lys contains one more methylene group than that of Orn. The solution structure of RA-42 has been determined by NMR and CD spectroscopy and it folds into a hairpin helix–loop–helix motif that dimerises to form a four-helix bundle.[1]

The design of RA-42 has been described in detail previously[1] and, in short, the helical sequences contain residues with helix propensity. The folded helical structures are further stabilised by residues capable of N- and C-terminal capping, salt bridge formation and helix dipole stabilisation. The formation of the hairpin motif and the dimerisation are driven by the hydrophobic interactions between the amphiphilic helices. The amino acid composition has been varied as extensively as possible to simplify the assignment of the ^{1}H NMR spectrum and two phenylalanines were incorporated at the lower end of helix II to function as NOE reporter groups and to generate chemical shift dispersion. The design of proteins and polypeptides has been reviewed recently.[6]

The histidine and the lysine residues were placed in a sequence of the peptide that folds into a helical conformation, and the reactivity towards *p*-nitrophenyl esters of the imidazole group of His-15 at pH 5.9 is larger than that of 4-methylimidazole partly due to the presence of the flanking lysine residue and partly due to the difference in dissociation constants between the two nucleophiles as described previously.[2] The initial step of the reaction is the attack by the unprotonated form of the histidine side chain on the ester to form an acyl intermediate under the release of *p*-nitrophenol. If the histidine residue is flanked by a lysine or an ornithine located four positions after (i, $i + 4$) or three positions before (i, $i - 3$) the histidine in the amino acid sequence, the acyl group is transferred in a fast intramolecular reaction to the unprotonated form of the flanking residue to form an amide and regenerate free histidine.[3] The mechanism of the site-selective functionalisation reaction between RA-42 and mono-*p*-nitrophenyl fumarate has been determined previously.[2]

The incorporation of the cofactor mimic into the folded polypeptide

The reduced form of the nicotinamide residue of the cofactor NADH is the source of hydride ions in the reduction of carbonyl groups to form the corresponding alcohols in alcohol dehydrogenases. In order to incorporate a covalently bound nicotinamide into the sequence of LA-42 the *p*-nitrophenyl

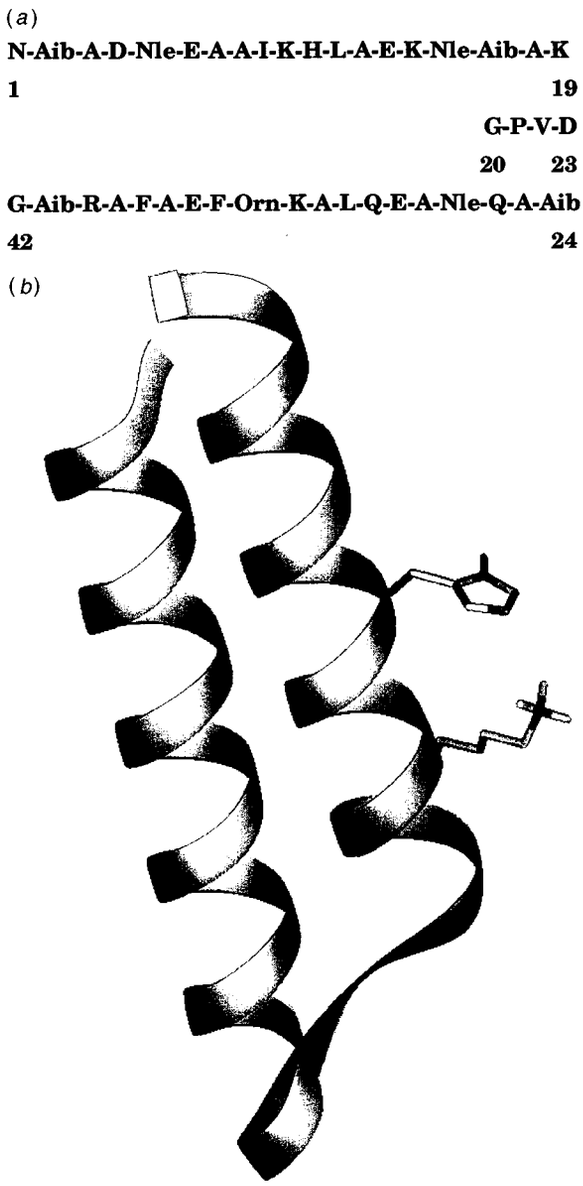


Fig. 1 The amino acid sequence (*a*) and modelled structure (*b*) of LA-42. The sequence is presented in the one letter code where A is Ala, D is Asp, E is Glu, F is Phe, G is Gly, H is His, I is Ile, K is Lys, L is Leu, N is Asn, P is Pro, Q is Gln, R is Arg, V is Val. Aib is α-aminoisobutyric acid and Nle is norleucine. Only the monomer of LA-42 is shown for simplicity and only the side chains of the residues in the reactive site.

ester of *N*-methylnicotinic acid **1** was synthesised and allowed to react with LA-42 in aqueous solution at pH 5.9, Fig. 2. The competing hydrolysis reaction was taken into account by using an excess concentration of ester that was calculated from the measured rate constants for the 'uncatalysed' hydrolysis and the 4-methylimidazole catalysed reactions. The second-order rate constant for the reaction between 4-methylimidazole and **1** was 5.5×10^{-3} dm^3 mol^{-1} s^{-1} and the observed pseudo first-order rate constant for the background hydrolysis reaction at pH 5.9 was 3.17×10^{-4} s^{-1}. The reactivity of LA-42 has previously been shown to be approximately five times higher than that of 4-methylimidazole in aqueous solution[3] so the estimated second-order rate constant of LA-42 was 2.75×10^{-2} dm^3 mol^{-1} s^{-1}. For a 0.8 mmol dm^{-3} concentration of LA-42 the pseudo-first order rate constant is 2.2×10^{-5} dm^3 mol^{-1} s^{-1} and *ca.* 7% of the ester will react *via* the peptide pathway, but as the reaction proceeds the concentration of LA-42 is diminished and a smaller fraction will be incorporated. The reaction was therefore carried out in two steps and the degree of nicotinoylation was checked with ES-MS. In the first incorporation 6 mg,

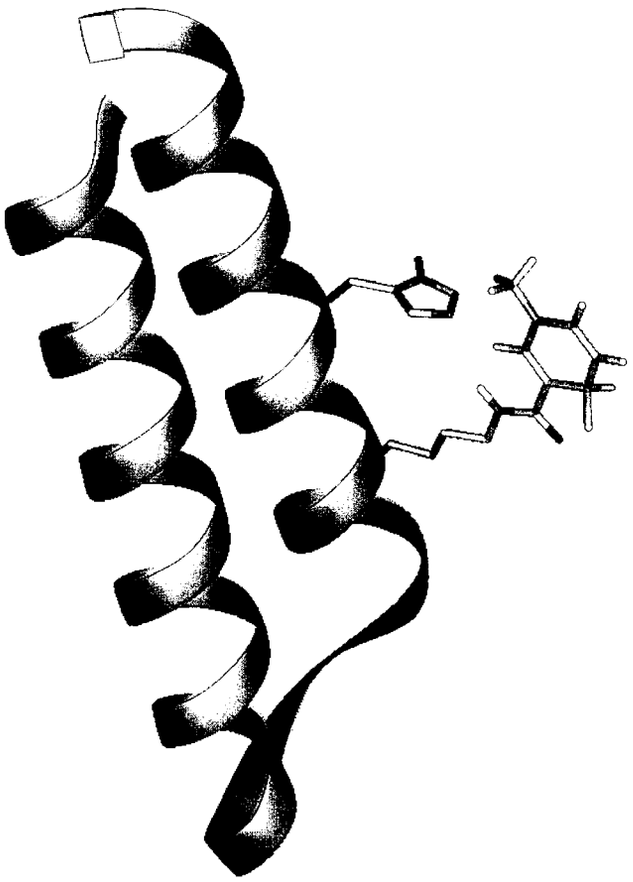


Fig. 2 Modelled structure of LA-42NAD

a thirteen-fold excess, of **1** was added to a solution of 5.2 mg of LA-42 (0.8 mmol dm^{-3}) in 1.5 cm^3 of aqueous sodium acetate buffer at pH 5.9.

The reaction was followed spectrophotometrically at 340 nm and when the release of *p*-nitrophenol was complete the reaction solution was analysed by LC-ESMS. The transformed mass spectrum showed one peak with a molecular weight of 4509.35 D corresponding to the weights of LA-42 (4390.10) and that of *N*-methylnicotinic acid (138.20) less that of water (18.02) and a second peak with a molecular weight of LA-42. No trace of diacylated product was observed. The ratio between the intensities in the mass spectrum suggested that *ca.* 60–70% of LA-42 had been acylated and the concentration of unmodified LA-42 was *ca.* 0.24 mmol dm^{-3}. LA-42 was therefore treated with an additional 8 mg of ester, a sixty-fold excess, and the excess amount needed was estimated from the ratio of the pseudo-first order rate constants, which was *ca.* fifty, calculated from the concentration of remaining LA-42, as described above. The discrepancy between calculated and obtained incorporation is due to the fact that the reactive peptide is consumed in the reaction because the flanking lysine is amidated. The decreased concentrations of unmodified LA-42 lead to pseudo first-order rate constants that are smaller and LA-42 competes less efficiently with the background reaction and the incorporation is incomplete.

After the reaction was finished the cofactor mimic bound LA-42, LA-42NAD, was purified by size exclusion chromatography using a Sephadex G-25 fine column with 0.1% trifluoroacetic acid as the eluent. LA-42NAD was obtained in a yield of 4.2 mg which is more than 70% based on the amount of LA-42 used as the starting material. The structure of LA-42NAD was investigated by NMR and CD spectroscopy and the mean residue ellipticity at pH 5.2 and room temperature was −18500 deg cm^2 $dmol^{-1}$ which is the same within experimental error as that of RA-42. The helical content had not therefore been reduced by the incorporation of the nicotinyl residue which is a necessary condition if the peptide is to be used as a template molecule.

The structure of the functionalised polypeptide

The 500 MHz ^{1}H NMR spectrum of LA-42NAD was assigned from the TOCSY and NOESY spectra recorded at 323 K in 5 vol% trifluoroethanol (TFE) in 90% H_2O/10% D_2O at pH 5.2 as described for RA-42 and guided by the assignment of the ^{1}H NMR spectrum of RA-42. The effect of TFE is to reduce amide proton exchange rates, increase the helical content of peptides with helix propensity and to disrupt the tertiary structure of proteins. At low concentrations of TFE the assignment of the ^{1}H NMR spectrum is considerably simplified due to the increased resolution but the supersecondary structure is not different from that in aqueous solution.[7]

The ^{1}H NMR chemical shifts of LA-42NAD are very close to those of RA-42 which shows that the structure of LA-42NAD is little affected by the bulky substituent. The chemical shifts of the amide protons of LA-42NAD are all within 0.1 ppm of those of RA-42 and the majority of the shifts are within 0.04 ppm of those of the unmodified peptide. There are no observable trends in the variation of NH chemical shifts *versus* position in the sequence.

The αH chemical shifts are used as indicators of secondary structure[8] and an upfield shift relative to those of random coil sequences are indicative of helical conformations. Downfield shifts show the formation of β sheet structures. The αH chemical shifts of RA-42[9] show the formation of helical segments between Ala-3 and Nle-16, and between Asp-23 and Gly-42, with a β-like loop sequence between Lys-19 and Gly-20.[8] The magnitude of the deviations from random coil values were as large as −0.44 ppm. The αH chemical shift deviations of LA-42NAD from random coil values were almost identical to those of RA-42 with amplitudes for 90% of the residues within 0.05 ppm of those of RA-42 and the remaining 10% within 0.12 ppm showing both larger and smaller deviations from those of RA-42. No observable trend was observed for variations in the αH chemical shifts and the secondary structure of LA-42NAD is therefore very similar to that of RA-42.

The formation of the hairpin motif is demonstrated by NOE connectivities between Phe aromatic protons and the methyl groups of Leu-12 or Ile-9 or both, as described for the helix–loop–helix dimer SA-42.[7] These long-range NOEs were observed for LA-42NAD which shows that the supersecondary structure, too, was unaffected by the incorporation of the *N*-methylnicotinoyl residue and that folded peptides function well as templates for the incorporation of complex functionality.

The incorporation of the nicotinoyl residue was also established by the observation of its spin system in the ^{1}H NMR TOCSY spectrum of LA-42NAD, Fig. 3. Three nicotinyl spin systems were observed. One of them showed well resolved resonances in the 1D spectrum and long relaxation times typical of small molecules, whereas two of the spin systems showed line broadening and relaxation times comparable to those of the resonances of LA-42NAD. The former spin system is therefore that of free *N*-methylnicotinic acid, which apparently comigrated with the peptide and was not removed by gel filtration, whereas the latter two are due to the covalently bound cofactor model. The reason that two spin systems are observed is probably due to restricted rotation around the amide bond which is well known to occur for amides *e.g.* in the case of dimethyl formamide. The chemical shifts tentatively assigned to the protons closest to the amide bond are shifted the most. No trace of incorporation of two nicotinoyl residues was found in the mass spectrum.

The reduction of the polypeptide bound NAD^+ cofactor mimic

The reduction of the nicotinoyl residue was carried out under nitrogen by 5–10 equiv. of sodium dithionite and disodium car-

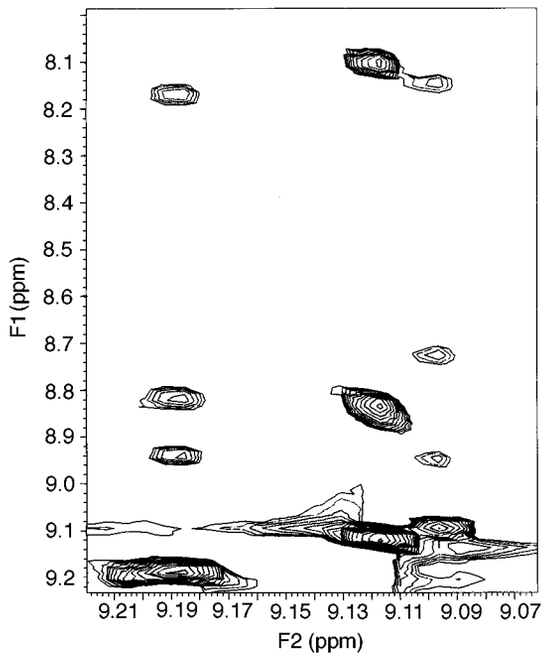


Fig. 3 Part of the ^{1}H NMR TOCSY spectrum of LA-42NAD in 5 vol% TFE in 90% H_2O/10% D_2O at 323 K and pH 5.1 showing the spin systems of the nicotinoyl residues. The spin system originating at 9.12 ppm shows well-resolved resonances with long relaxation times in the ^{1}H NMR spectrum, whereas the spin systems originating at 9.10 and 9.19 ppm show broadened resonances with relaxation times in the range of those of the peptide. No evidence for the formation of diacylated peptides were found in the mass spectrum and the reason for the formation of two spin systems is probably hindered rotation around the amide bond. The lower part of the spectrum shows the diagonal peaks and the distortions of *N*-methylnicotinic acid is an artifact.

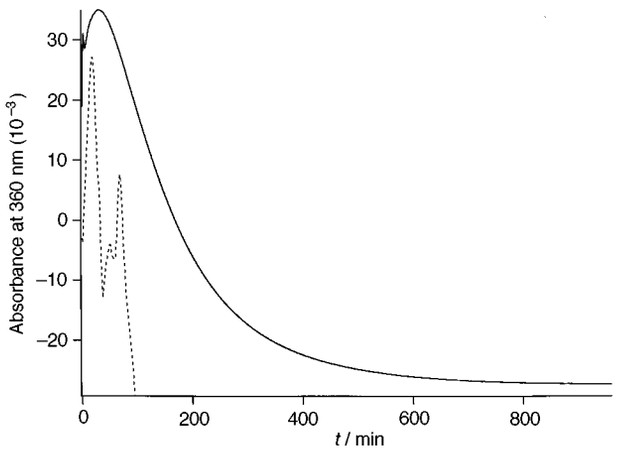


Fig. 4 UV absorbance in arbitrary units at 360 nm as a function of time showing the formation and degradation of the dihydropyridine upon reduction with sodium dithionite in aqueous solution at pH 7. The solid line shows the absorbance of the peptide-linked residue whereas the dashed line shows that of the reduced form of 1-methylnicotinamide. The absorbance of the solution containing reduced 1-methylnicotinamide is disturbed by precipitation but the lifetime of ornithine-linked nicotinic acid, which is soluble in both redox forms, is practically the same as that of the 1-methylnicotinamide derived product.

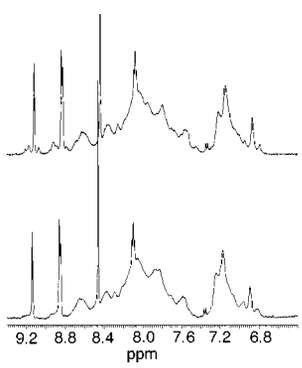


Fig. 5 Part of the 1D ^{1}H NMR spectrum of LA-42NAD showing the reduction of the *N*-methylnicotinoyl residue by sodium dithionite in D_2O solution at pH *ca.* 7 (uncorrected) and 293 K. The top trace shows the 1D spectrum before reduction and the bottom trace shows nearly complete reduction. After complete reduction no trace of nicotinoyl aromatic protons are observed (data not shown). The sharp resonances are those of a small impurity of *N*-methylnicotinic acid that comigrates with LA-42NAD on a size exclusion column. The linewidths of the resonances of the impurity are probably five to ten times smaller than those of the peptide-linked protons and the intensities of the resonances are therefore misleading in terms of concentrations. The resonances of the peptide-linked nicotinoyl protons have been assigned and their chemical shifts are shown in the TOCSY spectrum, Fig. 3. They are 9.10, 8.95, 8.72 and 8.13 ppm for one of the rotamers and 9.19, 8.94, 8.82 and 8.17 ppm for the other. Note that the peptide-linked nicotinoyl residue is reduced whereas *N*-methylnicotinic acid is not.

bonate in degassed aqueous solution at pH 7 and followed by NMR and UV spectroscopy. The reduction is readily carried out at low pH but the reduced dihydropyridine is quickly hydrolysed.[10] At high pH the reduction is slower or does not take place but the lifetime of the reduced form is longer and it was found that the optimum pH was 7. For comparison 1-methylnicotinamide was reduced, too, under identical experimental conditions. The lifetimes of the reduced forms of LA-42NAD and of 1-methylnicotinamide are shown in Fig. 4 where the absorption at 360 nm is due to the reduced nicotinyl residue. The dihydropyridine is known to have a limited life time due to the hydrolysis of the enamine and it is shown that the lifetime of the reduced LA-42NAD is several hours which is much longer than that of the reduced form of 1-methylnicotinamide. The reduced 1-methylnicotinamide is also poorly soluble and precipitates in aqueous solution.

The reduction of LA-42NAD was also followed by ^{1}H NMR spectroscopy in D_2O solution at pH 7 and 293 K. The pyridine spin system is readily detected in D_2O solution since the protons have chemical shifts in the region between 8.10 and 9.19 ppm and upon reduction this spin system disappears, Fig. 5. A small amount of *N*-methylnicotinic acid is also present as an impurity but is not reduced. The observation of the disappearance of the pyridine resonances by NMR spectroscopy therefore shows that the dihydropyridine is formed, in agreement with the observation from UV spectroscopy.

The enhanced lifetime of the enamine and the solubility in aqueous solution are important advantages of polypeptide linked cofactor mimics over the corresponding model systems soluble in organic solvents. The incorporation of the cofactor mimic into a folded polypeptide template allows the systematic exploitation of flanking residues capable of substrate recognition and transition state stabilisation and turnover is achievable in aqueous solution. The demonstration of the incorporation of a cofactor mimic into a folded motif offers a substantial advantage over the incorporation of protected amino acid derivatives, both in terms of the synthetic efforts needed to introduce complex functionality into enantiomerically pure non-natural amino acids and in terms of the synthetic efforts needed to succeed in the synthesis of the peptide considering the bulkiness of the protected amino acid derivative. The post-synthetic incorporation of achiral ester derivatives into folded motifs gives rise to enantiomerically pure functional groups since the amino acid residue to which the cofactor mimic is linked is chiral, and the synthetic efforts are therefore considerably smaller. Also, the flexibility provided by having access to a template motif which can be used for many different cofactors provides the opportunity of exploring screening techniques in the search for catalytic activity.

Conclusions

It has been shown that the incorporation of a NAD cofactor mimic into a folded polypeptide or protein is conveniently accomplished by adding the *p*-nitrophenyl ester of the cofactor model system to an aqueous solution of the peptide at pH 5.9. The postsynthetic and site-selective incorporation of cofactor functionality offers substantial advantages over the incorporation of enantiomerically pure amino acid derivatives in the peptide synthesis. The protein template makes the cofactor water soluble and it increases the lifetime of the dihydropyridine at pH 7 and room temperature by several hours, or more than a factor of three. The incorporation of residues in the reactive site for substrate recognition and transition state binding is now possible by minor modifications of the amino acid sequence and it facilitates *e.g.* the generation of small libraries of systematically modified sequences for screening purposes.

Experimental

Synthesis of *p*-nitrophenyl *N*-methylnicotinoate

p-Nitrophenyl nicotinoate was synthesised according to published procedures.[11] Nicotinic acid (0.522 g, 4.06 mmol), dicyclohexylcarbodiimide (0.965 g, 4.68 mmol) and *p*-nitrophenol (0.650 g, 4.67 mmol) were dissolved in 35 cm^3 of dichloromethane (DCM) and stirred at ambient temperature. 4-Pyrrolidinopyridine (63 mg, 0.4 mmol) in 4 cm^3 of DCM was added dropwise and the solution turned yellow. The reaction mixture was stirred for six days, treated with 4 cm^3 1.1 mol dm^{-3} HCl and filtered to remove precipitated dicyclohexylurea. The filtrate was washed with 30 cm^3 0.4 mol dm^{-3} HCl, 50 cm^3 of H_2O and dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to yield the crude product. After crystallisation from ethanol colourless needles were recovered in 92% yield (0.912 g). Mp 171 °C (lit., 172 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.41 (s, 1H), 8.91 (d, 1H), 8.47 (d, 2H), 8.36 (d, 2H), 7.51 (m, 1H), 7.45 (dd, 2H).

p-Nitrophenyl *N*-methylnicotinoate was synthesised according to published procedures.[12] *p*-Nitrophenyl nicotinoate (0.912 g, 3.74 mmol) was dissolved in 5 cm^3 dimethyl formamide and methyl iodide (0.798 g, 5.62 mmol) was added. The reaction mixture was stirred in darkness for six days at ambient temperature after which the solution had turned red, probably due to the presence of iodine. 200 cm^3 of diethyl ether was added and the product precipitated. Soxhlet extraction with diethyl ether as the solvent gave the pure product in almost quantitative yield. ^{1}H NMR (400 MHz, DMSO) δ 9.81 (m, 1H), 9.26 (d, 1H), 9.18 (d, 1H), 8.44 (d, 2H), 8.35 (t, 1H), 7.71 (d, 2H). FAB-MS, 259 calc. 259.072.

Peptide synthesis, purification and identification

LA-42 was synthesised using a PerSeptive 9050 Plus automated peptide synthesiser on a 0.1 mmol scale according to standard Fmoc protocols with HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] as the activating agent. A PAC-PEGPS polymer was used with a substitution level of 0.24 mmol g^{-1}. It was cleaved from the resin by treatment with a solution of 4.5 cm^3 trifluoroacetic acid, 250 mm^3 thioanisole, 150 mm^3 ethanedithiol and 100 mm^3 anisole for 20 min at 0 °C and 3 h at room temperature and precipitated by the addition of cold diethyl ether, centrifuged, resuspended three times in cold diethyl ether and then lyophilised. The crude peptide was purified by isocratic reversed-phase HPLC on a 20 × 250 mm Kromasil C-8 column using 42% isopropyl alcohol in 0.1% TFA as the eluent and then lyophilised. The purity was checked by analytical HPLC and a symmetric peak was found to contain only LA-42 by electrospray mass spectrometry using a VG ZabSpec magnetic sector mass spectrometer, found 4509.35, calc. 4510.28.

NMR spectroscopy

The ^{1}H NMR spectra were recorded on a Varian Unity 500 NMR spectrometer equipped with a matrix shim system from Resonance Research Inc. A 90° pulse of 9.2 μs and a temperature of 323 K were used for the TOCSY and NOESY spectra, which were recorded in 5 vol% TFE in 90% H_2O/10% D_2O in the phase-sensitive mode with a sweep width of 7000 Hz and 2 × 256 increments at pH 5.2. The spin-lock time of the TOCSY experiment was 60 ms and the mixing time of the NOESY experiment was 200 ms. The reduction of the nicotinoyl residue was followed at 293 K at pH *ca.* 7 (uncorrected) in D_2O solution. Relaxation measurements were carried out by the 180-τ-90 sequence to determine the null time of the nicotinoyl resonances.

CD spectroscopy

The CD spectra of LA-42NAD were recorded on a JASCO J-720 spectropolarimeter in the range from 300 to 190 nm. The instrument was calibrated with D-(+)-10-camphorsulfonic acid.

UV spectroscopy

UV spectra were recorded on a Cary 1 Bio UV-visible spectrophotometer equipped with a Cary temperature controller. Kinetics were measured at 290.2 K and pH 5.9 by following the absorbance of *p*-nitrophenol at 320 nm. The second-order rate constant of the 4-methylimidazole catalysed reaction was obtained from the slope of the linear plot of pseudo-first order rate constants *versus* concentration of 4-methylimidazole.

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