Protein Synthesis

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# Chemical Synthesis of the 20 kDa Heme Protein Nitrophorin 4 by α-Ketoacid-Hydroxylamine (KAHA) Ligation

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***Abstract:*** *The chemical synthesis of the 184-residue ferric heme-binding protein nitrophorin 4 was accomplished by sequential couplings of five unprotected peptide segments using α-ketoacid-hydroxylamine (KAHA) ligation reactions. The fully assembled protein was folded to its native structure and coordinated to the ferric heme b cofactor. The synthetic holoprotein, despite four homoserine residues at the ligation sites, showed identical properties to the wild-type protein in nitric oxide binding and nitrite dismutase reactivity. This work establishes the KAHA ligation as a valuable and viable approach for the chemical synthesis of proteins up to 20 kDa and demonstrates that it is well-suited for the preparation of hydrophobic protein targets.*

The chemical synthesis of proteins has emerged as a powerful tool for protein preparation and engineering as it enables complete control over the composition of a protein, including the synthesis of protein enantiomers and the site-specific incorporation of unnatural amino acids or post-translational modifications.[1] Until the advent and adoption of native chemical ligation (NCL), first utilized for protein synthesis by Kent and co-workers in 1994,[2] chemical protein synthesis was restricted to only a handful of examples requiring Herculean efforts to synthesize longer segments and couple them together.[3] NCL has enabled the preparation of dozens of proteins,[1,4] including examples over 300 residues in length.[5] Significant progress has been made to address some of the key issues in using NCL, including selective desulfurization reactions[6] and the development of new linkers to prepare peptide thioesters using Fmoc-based solid-phase peptide synthesis (SPPS; Fmoc = 9-fluorenylmethoxycarbonyl).[7] Nevertheless, the search for thiol-independent chemical reactions suitable for protein synthesis has been very attractive. Several methods, including the Staudinger ligation,[8] the α-ketoacid-hydroxylamine (KAHA) ligation,[9] and the serine/threonine ligation of peptide salicylate esters,[10] offer promising advances, but none other than NCL has yet been applied to the preparation of proteins larger than about 100 residues or on a significant (> 10 mg) scale.

The KAHA ligation, first reported by our group in 2006,[11] features a highly chemoselective ligation between two fully unprotected peptide segments bearing a C-terminal α-ketoacid or an N-terminal hydroxylamine. Recent advances in the KAHA ligation including: 1) the development of linkers for SPPS that deliver α-ketoacids directly upon resin cleavage,[12] 2) the identification of 5-oxaproline (5-Opr) which introduces a homoserine (Hse; $T^{\S}$) residue at the ligation site as a stable, yet sufficiently reactive, hydroxylamine in the KAHA ligation,[13] and 3) the use of Fmoc-protected 5-Opr and cyanosulfurylide as easily unmasked surrogates of the key functional groups in multiple segment ligations,[14] have greatly improved its applicability in chemical protein synthesis. The KAHA ligation has been successfully applied for the synthesis of various proteins up to about 100 amino acid residues, including Pup, cspA, UFM1, SUMO2/3, and S100A4.[12–15] A limitation of 5-Opr is the formation of a noncanonical Hse residue at the ligation site. Solutions to this include our recent disclosure of an oxazetidine amino acid that forms the natural serine residue upon ligation[15] or a careful selection of the ligation sites so that the Hse mutations do not affect protein structure or function. A convincing demonstration of the latter is particularly important as it is known that the related homocysteine residues result in unstable peptide chains which might lead to the formation of toxic multimers, amyloids, or aggregates.[16]

We now document the application of homoserine-forming KAHA ligations to the successful synthesis, folding, heme insertion, and NO binding of nitrophorin 4 (NP4), a 184-residue ferric heme-binding β-barrel protein. NP4 belongs to a family of at least five proteins, NP1–4 and NP7, originating from the saliva of the blood-sucking insect *Rhodnius prolixus*.[17] They are nitric oxide (NO) transporting proteins and a unique class of nitrite dismutase (EC 1.7.6.1).[18] The X-ray crystal structures of NP4 with various ligands have been solved to almost atomic resolution.[19] It is composed of an eight-stranded β-barrel, forming a lipocalin structure. The noncovalent heme *b* cofactor is coordinated to the protein through the His59 residue. The protein contains four Cys residues which, upon folding, form two disulfide bridges (Cys2–Cys122 and Cys41–Cys171). After the full-length peptide chain is assembled, we envisioned that it could be folded to its native apoprotein form followed by heme cofactor insertion to establish the holoprotein. The spectroscopically well-characterized heme cofactor served as an easy but powerful marker for the folding of the synthetic protein and for its biological function, specifically NO binding and nitrite dismutase activity, which can be assayed by absorption

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spectroscopy. Several known point mutations that completely disrupt the nitrite dismutase activity[18d,20] make this target ideal for probing any detrimental effects of the Hse residues.

To establish the convergent chemical synthesis of NP4 by KAHA ligations, we identified four disconnection sites at Leu44–Ala45, Leu74–Gln75, Phe107–Thr108, and Leu137–Asn138 based on the available α-ketoacid building blocks and the X-ray crystal structure (Figure 1; Figure S1 in the Supporting Information). The ligation sites were selected so that they did not fall into the active site, the heme pocket of NP4. As highlighted in Figure 1 B, all the resulting mutations (Ala45Hse, Gln75Hse, Thr108Hse, and Asn138Hse) are

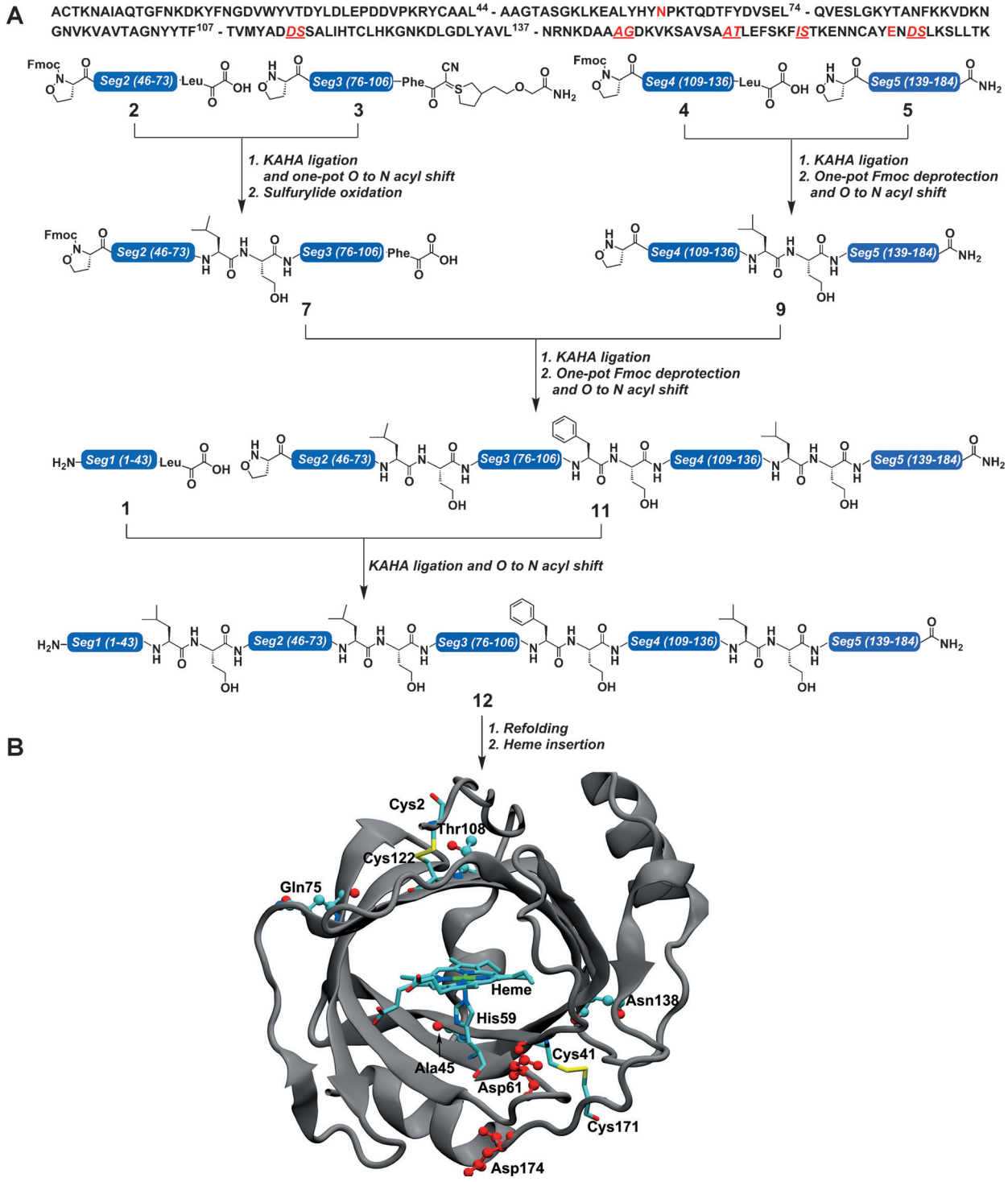


***Figure 1.*** Total chemical synthesis of NP4[T[S] 45, 75, 108, 138, D61N, D174E]. A) Amino acid sequence and convergent strategy. Dipeptide building blocks used during SPPS are in red and underlined. Two other mutations, introduced during the synthesis, Asn 61 and Glu 174, are highlighted in red. B) X-ray crystal structure of NP4 at pH 7.5 (PDB code: 1NP4), highlighting the four ligation sites (ball and stick representation), two disulfides (stick representation), and two other mutation sites for synthetic purposes (ball and stick representation, red).

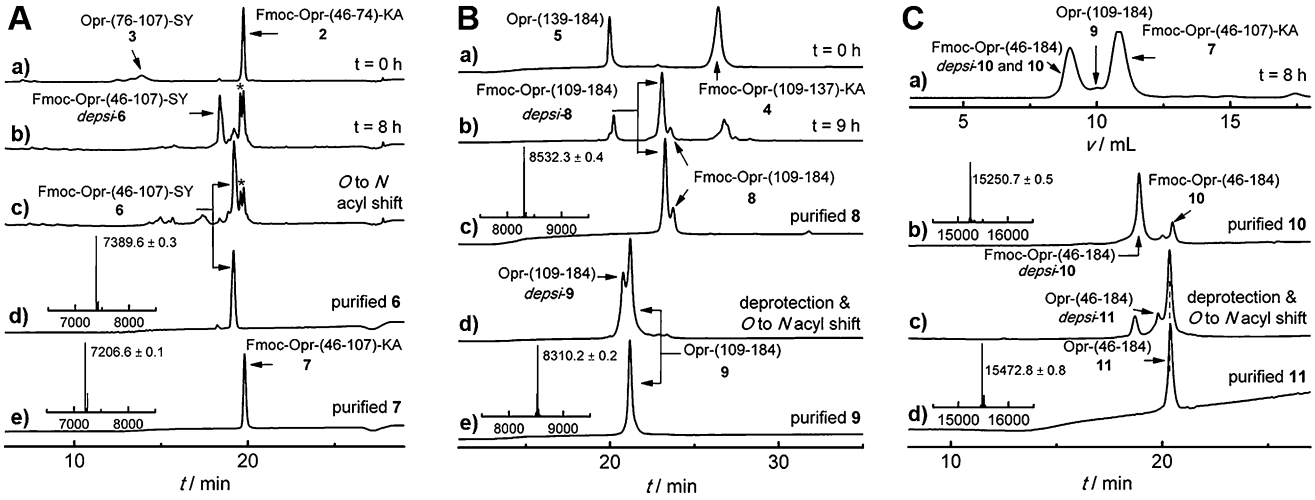


***Figure 2.*** Analytical HPLC traces ($\lambda = 210$ nm) for the convergent assembly of NP4 segments. A) KAHA ligation of **2** and **3**, followed by oxidation to **7**. B) KAHA ligation of **4** and **5**, followed by Fmoc deprotection to **9**. C) KAHA ligation of **7** and **9** as purified by SEC ($\lambda = 210$ nm), followed by Fmoc deprotection to **11**. The asterisk indicates the partially oxidized ketoacid segment **2**. KA = α-ketoacid; SY = sulfurylide. Insets in (A-d,e), (B-c,e), and (C-b,d): ESI-MS with units given in daltons (Da).

located more than 10 Å away from the heme iron. When modeled into the X-ray crystal structure, these mutations showed negligible effect on the overall structure (Figure S1). Unprotected peptide segments with C-terminal α-ketoacids can be derived either from oxidation of a cyanosulfurylide[21] or by acidic cleavage of the protected α-ketoacid.[12] Using these strategies, a wide range of α-ketoacids including Leu, Phe, Val, Ala, Arg, Glu, Ile, and Tyr are easily accessible. The hydroxylamine reaction partner was incorporated as 5-Opr at the N terminus of the requisite segments. For a convergent synthesis, the 5-Opr was conveniently protected as Fmoc, which survives both cleavage from the resin following SPPS and the ligation conditions and is easily removed under basic conditions. Cyanosulfurylides, robust and easily handled masked α-ketoacids, are formed directly upon resin cleavage using our previously documented linker.[21]

Details of the synthesis of peptide segments are provided in the Supporting Information. The use of pseudoproline dipeptides[22] or Dmb-backbone-protected Gly dipeptides[23] was crucial during the SPPS of NP4 segments **4** (NP4 108–137) and **5** (NP4 138–184) to achieve reasonable yields (Dmb = dimethoxybenzyl). Two other mutations were deliberately introduced during the synthesis (Figure 1B). The D174E mutation in NP4 segment **5** was selected to avoid aspartimide formation, which dominated in all attempts to synthesize the natural fragment. The D61N mutation in NP4 segment **2** was incorporated because of significant hydrolysis of the Asp–Pro linkage under the aqueous acidic conditions of ligation and purification, a known instability of peptide sequences containing this pair.[24] Notably, Asp61 is not conserved among all NPs; it is replaced with an Asn residue at this position for NP2, NP3, and NP7.

With all peptide segments in hand, we proceeded to assemble the full-length protein by sequential KAHA ligations. Segment **3** was ligated with α-ketoacid segment **2** under standard KAHA ligation conditions (DMSO/$H_2O$ at 60 °C). As shown in Figure 2A-b the reaction was essentially complete within 8 h with most of segment **3** consumed. The major side product was oxidation of α-ketoacid segment **2** (to form a product with a mass loss of 28 Da; indicated by an asterisk in Figure 2A-b,c). As previously reported, the major ligation product of KAHA ligation is the depsipeptide.[25] *Depsi*-**6** was conveniently rearranged to amide **6** at pH 8.0 in 2.5 h directly after the ligation (Figure 2A-c; 30% yield over two steps). Following purification and lyophilization, sulfurylide **6** was oxidized with oxone in $CH_3CN/H_2O$ with 0.1% trifluoroacetic acid (TFA) to obtain the corresponding α-ketoacid **7** (Figure 2A-e; 60% yield), ready for subsequent KAHA ligation.

KAHA ligation of segments **4** and **5** was performed using NMP/$H_2O$ (NMP = *N*-methyl-2-pyrrolidone) as solvent to avoid oxidation of the cysteine residues and TCEP (tris(2-carboxyethyl)phosphine) was added to maintain a reducing environment during the ligation. The ligation proceeded within 9 h to afford the product mainly as the ester, *depsi*-**8** (Figure 2B-b). The ligation mixture was purified by reverse-phase HPLC (RP-HPLC) and the ester and amide products were combined and lyophilized (Figure 2B-c; 50% yield). The N terminus of segment **4** bore an Fmoc-protected 5-Opr, which upon deprotection can be employed for another KAHA ligation. We performed the Fmoc deprotection and *O*-to-*N* acyl shift of the ligation product mixture **8** in a one-pot fashion. The Fmoc deprotection was carried out at 0 °C using *N*,*N*-diethylamine (5%) in NMP in the presence of 2-mercaptoethanol. It should be noted that the addition of 2-mercaptoethanol is essential for Cys-containing peptides as the unprotected Cys residues can trap the dibenzofulvene released during the Fmoc deprotection. After deprotection, the reaction mixture was diluted with Tris buffer (pH 9.0), with DTT (40 mM) added to prevent thiol oxidation. The *O*-to-*N* acyl shift was complete within 30 min affording the ligation product with a N-terminal 5-Opr **9** (Figure 2B-e; approx. 45% yield over two steps).

With the two large segments **7** and **9** prepared, we performed the convergent ligation under conditions similar to those described for the connection of segments **4** and **5**. The ligation was allowed to proceed for 8 h. Ligation product **10** eluted at a retention time close to both segments **7** and **9** using either C4 or C18 columns for RP-HPLC at various solvent gradients, making purification extremely difficult. However, product **10** (approx. 15 kDa) could be conveniently separated from the substrate segments **7** (approx. 7 kDa) and **9** (approx. 8 kDa) by size exclusion chromatography (SEC; Figure 2C-a), provided that the SEC was performed under denaturing conditions (6 M guanidine hydrochloride (Gnd·HCl), pH 5.0) to prevent peptide aggregation. The corresponding fractions were collected as a mixture of *depsi*-**10** and **10** and desalted using preparative HPLC (Figure 2C-b; 35% yield). The lyophilized product was subjected to one-pot Fmoc deprotection and an *O*-to-*N* acyl shift using the conditions described above for the ligation product **8** (Figure 2C-c). The Fmoc-deprotected *depsi*-**11** was readily rearranged to the amide **11** (Figure 2C-d) within 30 min with approximately 40% yield over two steps.

The final ligation between segments **1** and **11** was performed under similar conditions as described above for the ligation of segments **4** and **5**, using 3 equiv of **1** with respect to the more valuable segment **11**. After 8 h, the ligation mixture was diluted with Gnd·HCl buffer (6 M; pH 7.5) and the resulting solution was left overnight at 4 °C to induce the *O*-to-*N* acyl shift. Again, it was not feasible to use RP-HPLC for the purification as the full-length synthetic NP4 **12** was found to co-elute with segment **11**. SEC purification was carried out under denaturing conditions (6 M Gnd·HCl, pH 7.5). As shown in Figure 3A, the ligation product **12** eluted first as the major peak and the remaining segment **11** as a small shoulder. The collected fraction was predominantly the product **12** as evidenced by analytical RP-HPLC and ESI-MS (Figure 3B, 38% yield).

The full-length synthetic NP4 **12** was initially refolded by quick dilution, as reported previously.[19a,26] Although the folded protein could be obtained with this procedure, the yield was low (< 5%) because of precipitation; a well-known phenomenon for the refolding of NPs.[27] Although this is not an issue for the expressed proteins, as they are usually produced on a rather large scale, an improved procedure was essential for the refolding of synthetic NP4. We instead employed a high-salt refolding buffer (0.4 M L-(+)-arginine, 0.2 M Tris (tris(hydroxymethylaminomethane)), 0.1 M $(NH_4)_2SO_4$, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM GSH (glutathione), and 0.2 mM GSSG (glutathione disulfide; pH 8.4)), with which the refolding mixture was almost clear after overnight incubation at 4 °C. The folded protein was concentrated and purified by semipreparative HPLC. As expected, the folded protein with two disulfides eluted earlier than the unfolded full-length peptide chain (Figure 3C and Figure S11). The yield of the refolding improved to 48% with the high-salt refolding buffer. The ESI-MS of the folded protein showed a loss of 4 Da, in excellent agreement with the formation of two disulfide bonds. The circular dichroism (CD) spectrum shown in Figure 3D indicates that the folded protein adopts an antiparallel β-barrel structure,[28] in agreement with the reported X-ray structure.[19a,29]

The heme *b* cofactor was titrated into the folded synthetic NP4 according to a related procedure (Figure S12).[19a] The reconstituted holoprotein was purified by SEC (Figure S12). The UV/Vis electronic absorption spectrum of the synthetic holoprotein is essentially identical to the wild-type NP4, with a Soret absorption band at $\lambda = 404$ nm (Figure 3E).[19b] Upon addition of DEA/NO (diethylamine NONOate diethylammonium salt), a NO releasing compound, the absorption maximum shifted to $\lambda = 420$ nm, with the concomitant formation of two pronounced Q-bands at $\lambda = 533$ and 568 nm (Figure 3E).[19b,30] When left open to air for approximately 10 min at pH 7.5, the coordinated NO is released from the heme iron, as evidenced by the shift of the absorption maxima back to $\lambda = 404$ nm (Figure 3E). The data presented shows that synthetic NP4 is fully active as a NO transporter as it is able to bind and release NO.[17b] The CD spectra of synthetic NP4 and NP4[$Fe^{III}$-NO] were also recorded (Figure 3D), indicating an intact protein structure upon heme incorporation and NO binding. The nitrite dismutase activity of synthetic NP4 was also evaluated. Upon incubation with 1 mM nitrite at pH 6.5, the absorption maxima shifted towards $\lambda = 420$ nm, indicating the formation of the ferric nitrosyl complex, NP4[$Fe^{III}$-NO] (Figure S13). As

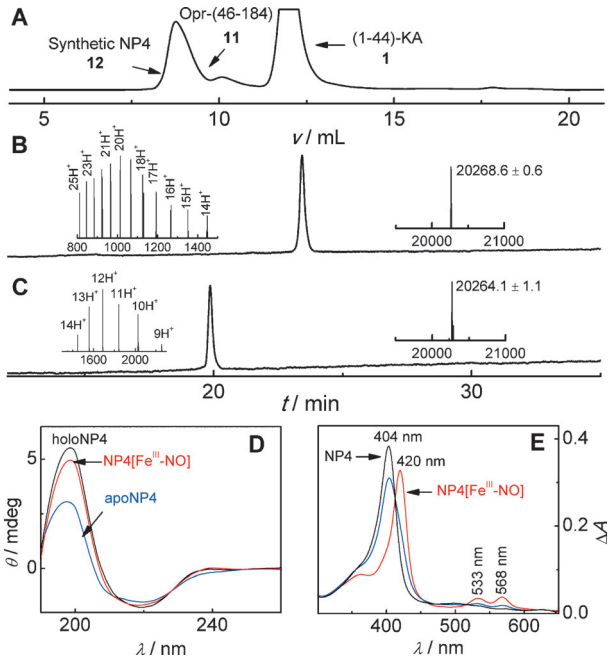


***Figure 3.*** Synthesis and folding of the full-length NP4 **12** and spectroscopic characterization. A) SEC ($\lambda = 280$ nm) purification profile of the final ligation after 8 h. Analytical HPLC trace ($\lambda = 210$ nm) of the ligation product **12** B) before and C) after refolding. Insets in (B) and (C) show ESI-MS, with units for the left insets given in *m*/*z* and the right insets in Da. From the ESI-MS, an observed mass of 20268.6 ± 0.6 Da (calcd. 20268.8 Da, average isotopes) and 20264.1 ± 1.1 Da (calcd. 20264.7 Da, average isotopes) were found for the denatured and folded protein, respectively. D) CD spectra of apoNP4 (blue), holoNP4 (black), and NP4[$Fe^{III}$-NO] (red). E) UV/Vis absorption spectra of NP4 (black), NP4[$Fe^{III}$-NO] (red), and NP4[$Fe^{III}$-NO] after 10 min in an open cuvette (blue).

  

such, the synthetic NP4 also retains the unique nitrite dismutase function.[18a,c]

In summary, the successful synthesis of NP4 on a multi-milligram scale establishes convergent KAHA ligations as a viable method for the preparation of synthetic proteins in the range of 20 kDa. It confirms that the KAHA ligation tolerates all canonical amino acids, including multiple cysteine residues. Although the reaction rate is slower than NCL, the KAHA ligation has features that make it very attractive for protein synthesis. First, the ligation conditions (aqueous, acidic NMP or DMSO) are ideal for solubilizing the peptide segments, particularly hydrophobic segments, such as those found in NP4 and related proteins. Second, many different α-ketoacid residues can be used, with Leu, by far the most common natural amino acids,[31] as one of the preferred choices. Third, both the hydroxylamine and the α-ketoacids can be easily masked, enabling many options for convergent ligations from multiple segments.

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