Semisynthesis of Bipyridyl–Alanine Cytochrome c Mutants: Novel Proteins with Enhanced Electron-Transfer Properties†

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Naturally occurring proteins possess enviable properties in that their diverse functions, accompanied by well defined supramolecular structures, provide ideal templates for the construction of new devices. Incorporation of nonencoded amino acids into protein ensembles serves to expand the opportunities well beyond the constraints set by nature. Methods for the generation of proteins containing unnatural amino acids include *in vitro* expression of chemically acylated suppressor tRNA,[1] total chemical synthesis,[2] and semisynthesis.[3] An example of the power of the semisynthetic approach for facile generation of multimilligram quantities is our construction of two horse heart cytochrome *c* (cyt *c*) mutants containing chelating π-acceptor amino acids, (*S*)-2-amino-3-(2,2′-bipyrid-6-yl)propanoic acid (6Bpa)[4] and (*S*)-2-amino-3-(2,2′-bipyrid-4-yl)propanoic acid (4Bpa),[5] at position 72 in the native protein sequence (Figure 1).[6] Because the bipyridyl ligands have a high affinity for metal cations[7] and can be placed site-specifically on the polypeptide backbone, they are ideal for building versatile electron-transfer (ET) model systems. Importantly, the bipyridyl amino acid mutations allow the assembly of a $Ru(bpy)_3^{2+}$ center[8] at a defined site on the protein surface, which is expected to increase markedly the efficiency of photoinduced ET from an electronically excited ($^*Ru^{2+}$) surface site to the protein metal center relative to that of a $Ru(bpy)_2(im)(His)$-modified protein.[9]

The mutant proteins were assembled through the coupling of two protein segments representing residues 1–65 and 66–104 of the complete horse heart cyt *c* primary sequence.[3] Native fragment 1–65, containing the covalently bound heme and a homoserine (Hse) lactone at the carboxy terminus, was obtained through cyanogen bromide cleavage of native horse heart cyt *c* at Met65, followed by purification of the heme-containing fragment using cation-exchange chromatography. Peptides 66–104, with the bipyridyl amino acids incorporated at position 72, were prepared by solid phase peptide synthesis, purified by reversed-phase HPLC, and characterized by electrospray mass spectrometry. Reconstitution of the protein was effected through incubation of the two purified fragments under neutral reducing conditions, thereby allowing reaction of the Hse65 lactone and the amino terminus of 66–104 to form a peptide bond. The renatured, fully formed protein was then purified using cation-exchange chromatography. The reconstitution reaction is extremely selective; reaction of crude peptide 66–104 with purified fragment 1–65 resulted in similar yields of protein in slightly lower purity. This remarkable selectivity is undoubtedly due to favorable secondary structure interactions between the two peptide fragments prior to amide bond formation. Characterization by several independent methods confirmed that incorporation of the bipyridyl amino acids does not perturb the three-dimensional structure of the protein.[10]

Modification of the 4Bpa and 6Bpa proteins with a redox-active unit was achieved via incubation with excess $Ru(bpy)_2CO_3$.[9] Singly modified derivatives were purified to homogeneity by cation-exchange chromatography. Bpa72-modified protein was unambiguously distinguished from the His33-modified form[9] by absorption and emission spectroscopy: $Ru(bpy)_3^{2+}$, $\lambda_{max}(abs)$ = 452 nm; $\lambda_{max}(em)$ = 615 nm (uncorrected); $Ru(bpy)_2(im)(His)^{2+}$, $\lambda_{max}(abs)$ = 492 nm; $\lambda_{max}(em)$ = 670 nm (uncorrected). Interestingly, only 4Bpa72 cyt *c* was modified at the surface bipyridyl residue (Figure 1). Molecular modeling of the two regioisomers indicates that the chelating nitrogens of the bipyridine are more accessible to solvent in the 4Bpa protein. Model peptide studies indicate that this regiospecificity cannot be attributed to an intrinsic property of the bipyridyl amino acids.[11]

Direct photoinduced (DP) and flash quench (FQ) techniques[9,12] were employed to obtain the rates of electron transfer in $Ru(bpy)_2$(4Bpa72)cyt *c*. The rates of both Fe^{2+}-to-Ru^{3+} ET and DPET ($^*Ru^{2+}$-to-Fe^{3+}) are much higher than those in the corresponding $Ru(bpy)_2(im)$-modified His72 protein(Table 1).[12,13] Since the intrinsic decay rates of the His72- and 4Bpa72-modified proteins are comparable, DPET rates are proportional to ET product yield. The increased yield of DPET products (~30%) in the 4Bpa72 protein relative to the His72 (2.3%)[12] protein may be understood in terms of the nature of the DPET reaction. The electron transfer originates from MLCT excitation, and the effective electron donor, a bipyridyl-based anion radical, is built directly into the polypeptide backbone in $Ru(bpy)_2$(4Bpa72)cyt *c*, in contrast to being at some undefined location with respect to the protein surface in $Ru(bpy)_2$-modified His72 cyt *c*. We conclude that the bipyridyl side chain enhances the distant donor-acceptor electronic coupling by effectively shortening the tunneling

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(10) Circular dichroism spectra of the mutant and native proteins are superposable. The UV–vis absorption spectra of the 4(6)Bpa72 and $Ru(bpy)_2$(4Bpa72) cyts *c* correspond exactly to the sum of their components. The reduction potentials of the mutant proteins are nearly identical with that of the native protein (native cyt *c* = 0.265(5) V, 6Bpa72 cyt *c* = 0.265(5) V, 4Bpa72 cyt *c* = 0.258(5) V *vs* NHE). Electrophoresis and chromatography data, EPR spectra, and thermospray mass spectra are all consistent with the proposed structure (see the supplementary material).

(11) Two peptides of the general sequence Ac-Bpa-Thr-Pro-D-Ala-Val-Phe-NH_2, where Bpa is either 4- or 6-substituted regioisomer, were synthesized and modified by $Ru(bpy)_2CO_3$ in high yield to afford derivatives with electronic absorption and steady-state emission properties characteristic of $Ru(bpy)_3^{2+}$. However, while the $^*Ru^{2+}$ lifetime of the $Ru(bpy)_2$(4Bpa) peptide (τ = 610 ns) is nearly identical with that of $Ru(bpy)_3^{2+}$ (τ = 640 ns), the $^*Ru^{2+}$ lifetime of the $Ru(bpy)_2$(6Bpa) peptide is significantly shorter (τ < 6 ns). The deactivation of the excited state in the 6Bpa derivative is attributed to a sterically induced increase in the Ru–N(6Bpa) bond length. Fujita, E.; Milder, S. J.; Brunschwig, B. S. *Inorg. Chem.* **1992**, *31*, 2079 and references therein.

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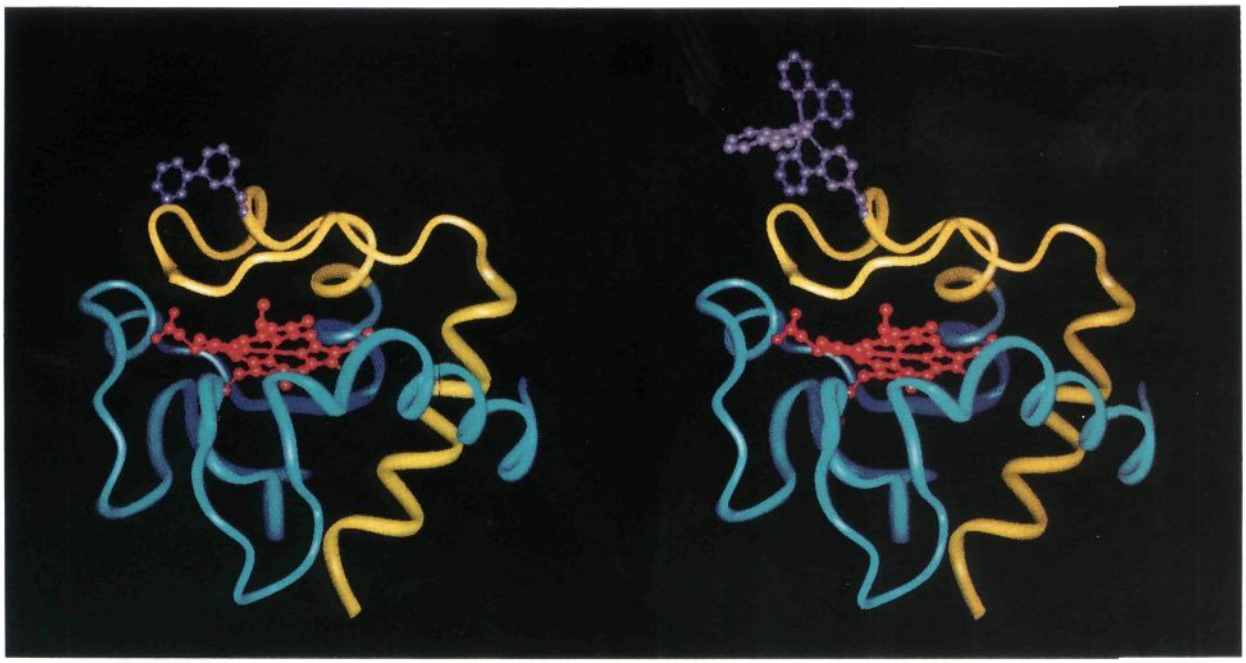

Figure 1. (left) Model of 4Bpa72 cyt *c* based on the crystal structure of ferric horse heart cyt *c*.[6] The blue tube is the 1–65 native fragment backbone; the yellow tube is the 66–104 backbone constructed by solid phase peptide synthesis; red indicates the heme group; and purple is the 4(6)Bpa72 residue. In the 6Bpa protein the chelating bipyridyl nitrogens atoms are facing into the protein; in the 4Bpa protein these nitrogens are solvent accessible. (right) Model of $Ru(bpy)_2$(4Bpa72)cyt *c* with purple indicating the $Ru(bpy)_3^{2+}$ unit.

Table I. Electron-Transfer Parameters for $Ru(bpy)_2$(4Bpa72)cyt *c*

	Excited-state lifetimes	ET Reactions			
	$*Ru^{2+}$ (ns)	$*Ru^{2+}$-to-Fe^{3+} (s^{-1})	$-\Delta G°$ [a]	Fe^{2+}-to-Ru^{3+} (s^{-1})	$-\Delta G°$ [a] (eV)
$Ru(bpy)_2$(4Bpa72)cyt *c* (pH 7.0)	Fe^{2+}:62 Fe^{3+}:52	$6(2) \times 10^6$	1.13	$6.5(5) \times 10^6$	1.00
$Ru(bpy)_2$(4Bpa72)cyt *c* (pH 11.0)	Fe^{3+}:51	$9(3) \times 10^6$	0.66	$1.2(5) \times 10^7$ $1.0(5) \times 10^6$	1.46
$Ru(bpy)_2$(His72)cyt *c* (pH 7.0)[12]	Fe^{2+}:70 Fe^{3+}:70	$3.4(7) \times 10^5$	1.17	$9.0(3) \times 10^5$	0.74

[a] Potentials for $Ru(bpy)_3^{2+}$ are used as models for $Ru(bpy)_2$(4Bpa72)cyt *c* since the ambient temperature emission and MLCT absorption properties of these two species are virtually identical. $Ru(bpy)_3^{*2+/3+} = -0.86$ V; $Ru(bpy)_3^{2+/3+} = 1.26$ V;[8] $Ru(bpy)_2(im)(His)^{*2+/3+} = -0.9$ V; $Ru(bpy)_2(im)_2^{2+/3+} = 1.0$ V;[9] cyt *c* = 0.265 V (pH 7); –0.2 V *vs* NHE (pH 11).[15]

pathway between the Ru and heme redox units in position-72-modified proteins.[12,14]

The enhanced ET properties of $Ru(bpy)_2$(4Bpa72)cyt *c* can be employed to probe conformationally perturbed states of the protein. At high pH ($pK_a \sim 9.3$), ferric cyt *c* exists in a low-potential state (the midpoint potential is roughly –0.2 V *vs* NHE) with altered ligation,[15] while under identical conditions the ferrous protein has the native structure. Owing to the fast photoinduced electron injection in $Ru(bpy)_2$(4Bpa72)cyt *c* at pH 11 (<60 ns, 480-nm excitation) (Table I), a high yield of the ferrous protein in the ferric alkaline conformation can be obtained.[16] The ability to photogenerate reduced cyt *c* rapidly with high quantum efficiency in conformationally altered forms opens the way for spectroscopic studies in the time domain (<microseconds) that is considered critical for the understanding of protein-folding dynamics.[17]

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Supplementary Material Available: Details of the peptide and protein synthesis, characterization, ruthenium modification, and ET studies (8 pages). Ordering information is given on any current masthead page.

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