Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 79–84

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

ARTIFICIAL METALLOENZYMES BASED ON PROTEIN CAVITIES: EXPLORING THE EFFECT OF ALTERING THE METAL LIGAND ATTACHMENT POSITION BY SITE DIRECTED MUTAGENESIS

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Received 21 September 1998; accepted 18 November 1998

Abstract: In an effort to construct catalysts with enzyme-like properties, we are employing a small, cavity-containing protein as a scaffold for the attachment of catalytic groups. In earlier work we demonstrated that a phenanthroline ligand could be introduced into the cavity of the protein ALBP and used to catalyze ester hydrolysis. To examine the effect of positioning the phenanthroline catalyst at different locations wthin the protein cavity, three new constucts — Phen60, Phen72 and Phen104 — were prepared. Each new conjugate was characterized by UV/vis spectroscopy, fluorescence spectroscopy, guanidine hydrochloride denaturation, gel filtration chromatography, and CD spectroscopy to confirm the preparation of the desired contruct. Analysis of reactions containing Ala-OiPr showed that Phen60 catalyzed ester hydrolysis with less selectivity than ALBP-Phen while Phen72 promoted this same reaction with higher selectivity. Reactions with Tyr-OMe were catalyzed with higher selectivity by Phen60 and more rapidly by Phen104. These results demonstrate that both the rates and selectivities of hydrolysis reactions catalyzed by these constructs are dependent on the precise site of attachment of the metal ligand within the protein cavity.

The design of molecules that mimic the catalytic properties of naturally occurring enzymes is a significant challenge and the ability to design and prepare them would be useful for a variety of applications in chemistry and biology.[1] In an effort to construct such molecules, we are employing a small, cavity-containing protein as a scaffold for the attachment of catalytic groups. This strategy exploits the flexibility afforded by a protein cavity, that can be manipulated via site directed mutagenesis, to modulate catalyst reactivity.[2–4] Fatty acid binding proteins are a class of small, ca. 15 kDa, proteins with a predominantly β-sheet structure that encloses a large solvent sequestered cavity where hydrophobic ligands bind; detailed structural information exists for many members of this protein family.[5] In earlier work, we prepared an artificial metalloprotein from ALBP (Adipocyte Lipid Binding Protein) by alkylation of a unique cysteine residue (Cys_{117}) with a 1,10-phenanthroline-derived reagent. The resulting conjugate denoted as ALBP-Phen bound Cu(II) ions via the phenanthroline ligand and catalyzed the enantioselective hydrolysis of amino acid esters.[6,7] Since the rate and enantioselectivity of this hydrolytic reaction should be influenced by the environment around the catalytic metal center, we were interested in positioning the phenanthroline ligand at several different locations within the cavity to investigate this issue. To accomplish this, three mutants of a related protein, IFABP (Intestinal Fatty Acid Binding Protein) were employed. Each of these mutant proteins, V60C, L72C and A104C, contains a unique cysteine residue that can be used to attach the phenanthroline.[8] Here we report on the preparation and physical and catalytic characterization of three of such phenanthroline conjugates denoted as Phen60, Phen72, and Phen104.

0960-894X/99/$ - see front matter
PII: S0960-894X(98)00684-2

Phenanthroline conjugates were prepared by reacting the purified mutant proteins with iodoacetamido-1,10-phenanthroline (1) in HEPES buffer (25 mM, pH 7.5) at room temperature in the dark for 48 h as summarized in Figure 1.[6,9] During this time, the extent of cysteine alkylation was assessed by thiol titration with DTNB. The crude protein product from each conjugation reaction was purified by gel filtration chromatography to remove unincorporated phenanthroline. Each new conjugate was characterized by UV/vis spectroscopy, fluorescence spectroscopy, guanidine hydrochloride denaturation, gel filtration chromatography, and CD spectroscopy. Some representative data for one mutant, Phen72, is shown in Figure 2 and data for all three conjugates is summarized in Table 1.

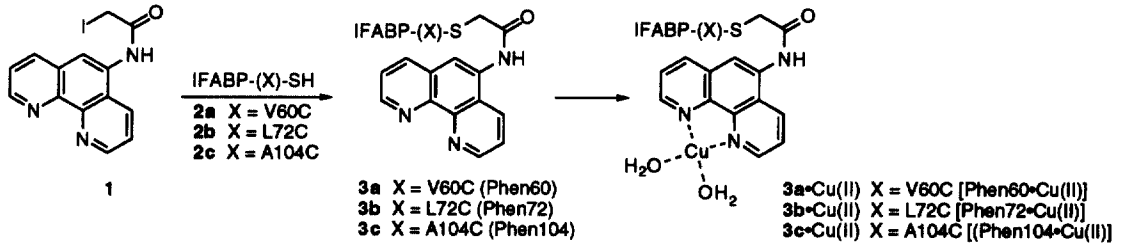


Figure 1. Procedure employed for the preparation and metallation of IFABP-phenanthroline conjugates.

UV/vis spectroscopy of all of the phenanthroline conjugates reveals a blue shift in λ_{max} and an increase in extinction (relative to the unmodified proteins) near 280 nm, which is consistent with the attachment of a phenanthroline moiety whose chromophore overlaps with protein-derived tyrosine and tryptophan residues; a shoulder centered near 320 nm also appears that can be assigned to the phenanthroline. These spectral features are shown in Figure 2A for Phen72. Spectra 1 and 2 are of L72C before and after modification while spectrum 3 is a difference spectrum that clearly shows the presence of a phenanthroline moiety. Fluorescence spectroscopy of the phenanthroline conjugates shows that substantial quenching of the intrinsic protein fluorescence occurs; for example, Figure 2B illustrates the emission spectra of L72C before (spectrum 1) and after (spectrum 2) phenanthroline attachment. These results suggest that the phenanthroline heterocycle is positioned within the cavity and is quenching the fluorescence of neighboring tyrosine and tryptophan residues that are also present within the cavity. The fluorescence emission spectrum of Phen72, shown in Figure 2B (spectra 2 and 3), also contains a shoulder centered at 420 nm due to the phenanthroline chromophore similar to that observed with ALBP-Phen. In contrast, this shoulder is considerably less marked in Phen60 and Phen104, presumably due to quenching by protein-derived side chains. Both the unmodified mutant proteins and their respective phenanthroline conjugates exhibit significant changes in their fluorescence emission spectra including shifts in λ_{max} and decreases in fluorescence intensity spectra upon denaturation (see Figure 2B, spectrum 4). These changes were used to assess the stability of these proteins in guanidine hydrochloride denaturation titrations. Attachment of phenanthroline to V60C or A104C appears to have little effect on the stability of these proteins. In contrast, conjugation of L72C with phenanthroline results in a substantial decrease in protein stability; the midpoint of the guanidine hydrochloride denaturation titration drops from 1.29 M to 1.08 M when L72C is modified (see Table 1). These trends in stability are similar to those observed with IFABP conjugates dansylated at the same positions.[8b] All of the phenanthroline conjugates appear to be well folded and maintain the overall β-sheet structure of IFABP as judged by gel filtration chromatography and CD spectroscopy. The modified proteins

chromatographed with comparable elution volumes as the unmodified precursors (see Table 1) and CD spectra of the conjugates retain substantial ellipticity at 220 nm characteristic of a β-sheet protein; circular dichroism spectra of L72C before (spectrum 1) and after (spectrum 2) phenanthroline attachment are presented in Figure 2C.

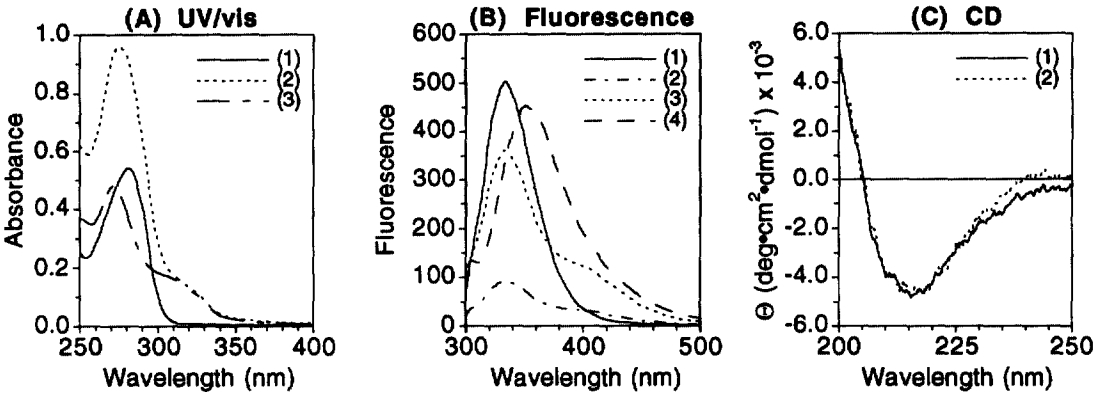


Figure 2. Spectroscopic properties of the L72C (**2b**) and Phen72 (**3b**) proteins. (A) UV/vis spectra obtained using 25 μM protein in Tris buffer (50 mM, pH 7.4). (1) Spectrum of L72C protein. (2) Phen72. (3) Difference spectrum of (2) minus (1). (B) Fluorescence spectra obtained using 1.0 μM protein in Tris buffer (50 mM, pH 7.4) at 25 °C with λ_{ex} = 275 nm and slit widths of 5.0 nm (excitation) and 4.0 nm (emission). (1) Emission spectrum of L72C protein. (2) Phen72. (3) Same as (2) but with slit widths of 7.5 nm (excitation and emission). (4) Same as (3) but obtained in 2.5 M guanidine•HCl. (C) Circular dichroism spectra obtained using 15 μM protein in Tris buffer (50 mM, pH 7.4). (1) Spectrum of L72C protein. (2) Phen72.

Table 1. Physical and spectroscopic properties of IFABP-phenanthroline conjugates.

Protein	λ_{max} [a] (nm)	ε_{280} [b] (cm^{-1}•mM^{-1})	Fluorescence[c] (%)	Denaturation Midpoint (M)[d]	Molecular Mass (kDa)[e]
V60C	282	19.0	-	1.19	18.6
Phen60	272	35.5	40	1.19	21.5
L72C	282	21.6	-	1.29	18.7
Phen72	276	37.3	19	1.08	20.6
A104C	282	17.2	-	1.66	18.7
Phen104	278	32.9	79	1.61	N.D.

[a]λ_{max} values obtained by UV/vis spectroscopy in Tris buffer (50 mM, pH 7.4). [b]Extinction coefficients at 280 nm determined by UV/vis spectroscopy as noted above. [c]Fluorescence emission at 335 nm (excitation at 275 nm) expressed as a percentage relative to the unmodified protein. [d]Midpoint of an equilibrium denaturation titration with guanidine hydrochloride. Titrations were performed using 0.5 - 1.0 μM protein in Tris buffer (50 mM, pH 7.4) and EDTA (100 μM) with variable concentrations of denaturant and were monitored by fluorescence emission (excitation at 280 nm). Denaturation midpoints were obtained by analysis of plots of fluorescence intensity or emission maximum versus guanidine hydrochloride concentration. [e]Native molecular masses were determined by gel filtration chromatography in HEPES (25 mM, pH 7.5) and NaCl (150 mM) using a Superose 12 HR 10/30 FPLC column (Pharmacia) at 4 °C and employing bovine lung aprotinin, horse heart cytochrome *c*, bovine erythrocyte carbonic anhydrase and bovine serum albumin as standards. N.D.: Not determined.

In earlier work with ALBP-Phen, the ability of this conjugate to bind Cu(II) ions was examined by fluorescence spectroscopy.[6] For that protein, it was found that the fluorescence spectrum contained two types of fluorophores; tyrosine and tryptophan residues emitted characteristically at 340 nm while the phenanthroline emitted at longer wavelengths and appeared as a shoulder centered near 410 nm. Addition of Cu(II) to ALBP-Phen resulted in extensive quenching of both the phenanthroline (410 nm) fluorescence as well as the protein fluorescence (340 nm). Unfortunately, the fluorescence properties of the IFABP-based constructs reported here

were less amenable to this type of analysis. Only Phen72 exhibited significant phenanthroline fluorescence as can be seen in the shoulder near 410 nm in spectrum 3 in Figure 2B. Figure 3A shows fluorescence spectra in the absence and presence of Cu(II); a fluorescence difference spectrum is presented in Figure 3B. These data clearly show that the phenanthroline fluorophore is perturbed by the added metal ion. A plot of protein (340 nm) and phenanthroline (410 nm) fluorescence versus equivalents of Cu(II), given in Figure 3C, shows that a slight increase in protein fluorescence accompanied by a significant diminution of phenanthroline fluorescence occurs upon addition of one equivalent of Cu(II); further addition of Cu(II) up to ten equivalents results in less pronounced decreases in both protein and phenanthroline fluorescence. These data provide good evidence for the binding of Cu(II) to the phenanthroline moiety of Phen72. Experiments with Ni(II), Zn(II), Co(II) and Fe(II) give similar results indicating that Phen72 is capable of binding a variety of metal ions. Similar experiments with Phen60 and Phen104 were inconclusive since these conjugates possessed no discernable phenanthroline fluorescence.

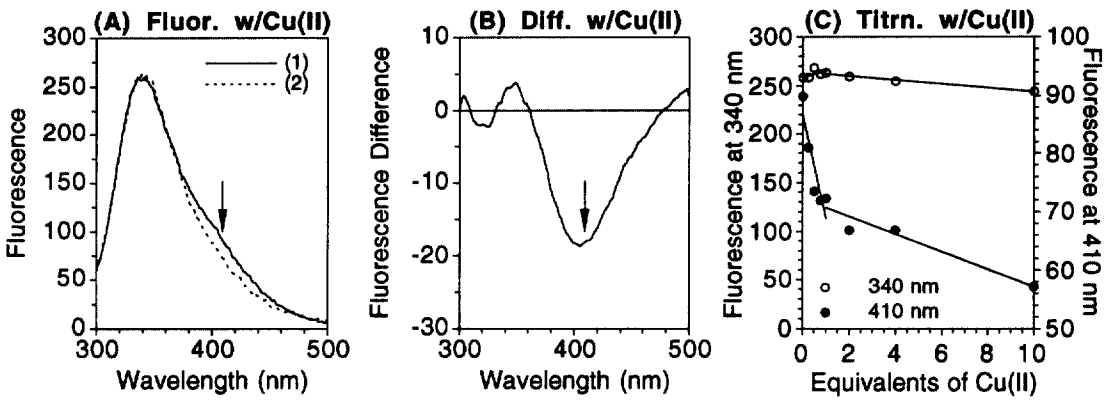
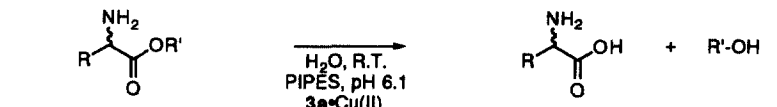


Figure 3. Titration of Phen72 conjugate with Cu(II) monitored by fluorescence spectroscopy. (A) Fluorescence spectra of Phen72 in the absence and presence of Cu(II). (1) Emission spectrum of Phen72. (2) Phen72 with one equivalent of Cu(II). Spectra were obtained using 0.5 μM protein in Tris buffer (50 mM, pH 7.4) at 25 °C with λ_{ex} = 275 nm and slit widths of 6.0 nm (excitation) and 9.0 nm (emission). The arrow highlights the phenanthroline shoulder at 410 nm. (B) Difference fluorescence spectrum obtained by subtracting spectrum (2) from spectrum (1). The arrow highlights the decrease in phenanthroline fluorescence upon Cu(II) binding. (C) Titration of Phen72 with Cu(II) monitored by fluorescence spectroscopy at 340 nm (o) and 410 nm (•). Lines have been added to emphasize the trends in the data. Spectral data was obtained as described in Part (A).

In earlier work with ALBP-Phen, the substrate Ala-OiPr (**4**) was hydrolyzed to Ala (**5**) with the high selectivity (85% *ee*) while Tyr-OMe (**6**) was hydrolyzed to Tyr (**7**) rapidly but with little selectivity.[6] Thus, we elected to study these hydrolysis reactions with the constructs Phen60, Phen72 and Phen104 as summarized in Figure 4. Reactions were performed using conjugate (25 μM) and racemic amino acid ester (1.0 mM) in PIPES buffer (10 mM, pH 6.1) at 25 °C. The production of the enantiomeric amino acid products was determined by reaction of the crude reaction mixture with *o*-diphthalaldehyde to form diastereomeric isoindole derivatives which were then separated by reversed phase HPLC and quantified by fluroescence detection.[10] Previous work with ALBP-Phen showed that this conjugate catalyzed the hydrolysis of Ala-OiPr under the conditions noted above with a rate of 1.4 μM/h and a selectivity of 85% *ee*. Experiments with Phen60 revealed that this conjugate catalyzed Ala-OiPr hydrolysis with a rate of 1.6 μM/h and a selectivity of 61% *ee*; thus the reaction was slightly faster than ALBP-Phen although the enantioselectivity decreased from 12:1 to 4.1:1. Experiments with Phen 104

indicated that this conjugate catalyzed Ala-OiPr hydrolysis with a rate of 0.54 μM/h and a selectivity of 83% *ee*. Hence, the selectivity of Phen104 is comparable to that of ALBP-Phen although the rate is 2.6-fold slower. In contrast, Phen72 catalyzes Ala-OiPr hydrolysis with a rate of 1.3 μM/h and a selectivity of 94% *ee*. Thus, Phen72 catalyzes ester hydrolysis at a rate similar to that measured for ALBP-Phen. However, the selectivity obtained with Phen72, 94% *ee* (32:1 enantiomeric ratio) is significantly higher than that observed for ALBP-Phen (85% *ee*, 12:1 enantiomeric ratio). Experiments with the substrate Tyr-OMe also produced interesting results; in general, the rates with this substrate were faster although the selectivities were poorer. Phen60 catalyzed the hydrolysis of Tyr-OMe with a rate of 4.0 μM/h and a selectivity of 61% *ee*. This rate is 1.6-fold slower that that obtained with ALBP-Phen although the selectivity is somewhat higher (61% *ee* versus 44% *ee*). In contrast, Phen104 catalyzed the Tyr-OMe hydrolysis with a rate of 11 μM/h and a selectivity of 46% *ee*. In this case, the hydrolysis rate is greater (1.7–fold) than that obtained with ALBP-Phen while the selectivities are comparable. Finally, it should be noted that no Tyr-OMe hydrolysis above background was observed using Phen72.



4 R = CH_3, R' = $(CH_3)_2CH$
6 R = $HOC_6H_5CH_2$, R' = CH_3

5 R = CH_3
7 R = $HOC_6H_4CH_2$

Figure 4. Hydrolysis reactions catalyzed by protein-phenanthroline conjugates based on ALBP and IFABP.

Table 2. Rates and enantioselectivities of ester hydrolysis reactions catalyzed by protein-phenanthroline conjugates Phen60 (**3a**), Phen72 (**3b**), Phen104 (**3c**) and ALBP-Phen.

Substrate / Product	Ala–OiPr (**4**) / Ala (**5**)				
Conjugate	D-Enantiomer Rate (μM/h)	L-Enantiomer Rate (μM/h)	Enantiomeric Ratio (L/D)	Enantiomeric Excess (%)	Turnovers after 24 h
Phen60 [**3a**•Cu(II)]	0.26	1.3	4.1	61	1.5
Phen72 [**3b**•Cu(II)]	0.04	1.3	32	94	1.3
Phen104 [**3c**•Cu(II)]	0.05	0.49	11	83	0.5
ALBP-Phen	0.10	1.3	12	85	1.3
Substrate / Product	Tyr-OMe (**6**) / Tyr (**7**)				
Conjugate	D-Enantiomer Rate (μM/h)	L-Enantiomer Rate (μM/h)	Enantiomeric Ratio (L/D)	Enantiomeric Excess (%)	Turnovers after 24 h
Phen60 [**3a**•Cu(II)]	0.72	3.3	4.1	61	3.8
Phen72 [**3b**•Cu(II)]	< 0.02	< 0.02	-	-	-
Phen104 [**3c**•Cu(II)]	3.0	8.0	2.7	46	10.6
ALBP-Phen	2.0	4.5	2.6	44	6.2

The results reported here demonstrate that both the rates and enantioselectivities of hydrolysis reactions catalyzed by phenanthroline•Cu(II) conjugates are dependent on the precise site of attachment of the metal ligand to the protein. While significant differences were obtained amongst the various phenanthroline conjugates, the range of effects observed here appears to be smaller than that noted in a similar study of pyridoxamine conjugates prepared from the same set of mutants (V60C, L72C, A104C).[3b] One reason for this behavior may be that the phenanthroline systems are conformationally mobile within the protein cavity. Such mobility is apparent for the phenanthroline ligand in ALBP-Phen whose crystal structure has recently been reported.[11] In that structure, all interactions between the protein and the phenanthroline (except the covalent attachment point) are nonbonded Van de Waals contacts. This can be contrasted with the structure of ALBP-PX, a pyridoxamine conjugate, where the

cofactor is conformationally locked due to hydrogen bonding interactions with the protein. Thus, it is likely that the hydrolysis reactions catalyzed by phenanthroline conjugates are occuring via a complex ensemble of conformations. It may be possible to reduce this conformational mobility by introducing protein-derived ligands to lock the metal in place. Such constructs, as well as others that incorporate additional functional groups to participate in catalysis are currently being prepared.

Acknowledgments. We thank Dr. C. Frieden and Dr. D. Bernlohr for providing the plasmids encoding the proteins used in this study. R. Davies was supported by a National Institutes of Health training grant (NIH 2T32GM08347-06). This work was supported by a grant from the National Science Foundation (NSF-CHE-9506793).

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