Supporting Information

Binuclear Cu_A Formation in Biosynthetic Models of Cu_A in Azurin Proceeds via a Novel $Cu(Cys)_2His$ Mononuclear Copper Intermediate

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Table S1: Rates derived from analysis of the O_2-rich stopped-flow data of all Glu114XCu_AAz variants.

Cu_A Variant	**Kinetic Model**	**Rate Constants** O_2**-rich**
Glu114GlyCu_AAz		$k_1 = 10.75 \pm 0.37\ s^{-1}$ $k_2 = 2.20 \pm 0.03\ s^{-1}$ $k_3 = (1.13 \pm 0.03) \times 10^{-3}\ s^{-1}$
Glu114AlaCu_AAz	$CuSO_4$ → T2 Cu T2 Cu → I_x I_x → T1 Cu	$k_1 = 19.72 \pm 1.27\ s^{-1}$ $k_2 = 0.47 \pm 0.03\ s^{-1}$ $k_3 = (2.18 \pm 0.05) \times 10^{-3}\ s^{-1}$
Glu114LeuCu_AAz		$k_1 = 11.61 \pm 0.75\ s^{-1}$ $k_2 = 0.35 \pm 0.005\ s^{-1}$ $k_3 = (2.13 \pm 0.04) \times 10^{-3}\ s^{-1}$
Glu114GlnCu_AAz		$k_1 = 6.77 \pm 0.41\ s^{-1}$ $k_2 = 0.33 \pm 0.01\ s^{-1}$ $k_3 = (3.41 \pm 0.06) \times 10^{-3}\ s^{-1}$

Table S2: Parameters extracted from fitting of the EPR data of Glu114XCu_AAz variants obtained at various time points after mixing apo proteins with 0.4 eq. $CuSO_4$. Parameters corresponding to the earliest time points are shown in Table 2.

Cu_A Variant	**Parameters**						**Populations (%)**					
		I_{x1}	I_{x2}	T1	T2	Cu_A	Time (min)	I_{x1}	I_{x2}	T1	T2	Cu_A
Glu114GlyCu_AAz	g_x	2.034	2.027	1.989	N/A	2.010	0.83	38	46	0	N/A	16
	g_y	2.036	2.020	2.048		2.026	35	35	44	12		9
	g_z	2.152	2.185	2.242		2.169	135	30	31	29		10
	$\|A_x\|$x10^{-4} cm^{-1}	10	23	22		21,19	285	17	16	45		22
	$\|A_y\|$x10^{-4} cm^{-1}	6	22	6		29,4	430	7	5	59		29
	$\|A_z\|$x10^{-4} cm^{-1}	101	87	69		63,57	1440	3	3	39		56
Glu114AlaCu_AAz	g_x	2.036	2.039	2.035	2.043	2.016	0.83	41	45	0	0	14
	g_y	2.022	2.022	2.045	2.095	2.026	5	36	40	5	3	15
	g_z	2.163	2.187	2.269	2.255	2.167	20	36	31	10	7	15
	$\|A_x\|$x10^{-4} cm^{-1}	7	12	9	31	19,23	60	20	14	24	19	22
	$\|A_y\|$x10^{-4} cm^{-1}	14	24	6	37	30,3	180	8	3	33	25	31
	$\|A_z\|$x10^{-4} cm^{-1}	100	75	45	142	62,57	270	7	2	31	28	32
							1320	4	0	29	25	42
Glu114LeuCu_AAz	g_x	2.009	2.005	2.040	N/A	2.014	0.75	55	22	0	N/A	23
	g_y	2.035	2.032	2.047		2.026	1	61	28	1		10
	g_z	2.163	2.240	2.259		2.168	5	54	24	6		15
	$\|A_x\|$x10^{-4} cm^{-1}	14	27	2		15,19	45	34	16	38		12
	$\|A_y\|$x10^{-4} cm^{-1}	12	13	27		31,6	210	3	2	80		14
	$\|A_z\|$x10^{-4} cm^{-1}	73	74	46		61,57						
Glu114GlnCu_AAz	g_x	2.011	1.991	2.031	N/A	2.018	0.83	68	18	3	N/A	10
	g_y	2.035	2.092	2.060		2.024	5	52	13	25		10
	g_z	2.151	2.249	2.289		2.171	20	35	9	33		23
	$\|A_x\|$x10^{-4} cm^{-1}	20	8	1		25,23	55	17	2	51		30
	$\|A_y\|$x10^{-4} cm^{-1}	14	41	23		31,7	225	11	2	47		40
	$\|A_z\|$x10^{-4} cm^{-1}	82	133	38		61,55						

Table S3: EPR Parameters of holo Cu_A for all variants prepared by mixing apo proteins with a mixture of 0.8eq Cu(II)/0.8 eq. Cu(I), shown in Figure S3. The experimental data were simulated as two types of Cu_A, one with symmetric, and another with asymmetric geometry. The parameters and relative population of the asymmetric Cu_A type are shown as italics in blue.

Cu_A Variant	**Parameters**			**Populations (%)**
Glu114GlyCu_AAz	g_x	2.014	*2.003*	57, 43
	g_y	2.019	*2.028*	
	g_z	2.167	*2.212*	
	$\|A_x\|$x10^{-4} cm^{-1}	24, 16	*32, 13*	
	$\|A_y\|$x10^{-4} cm^{-1}	19, 6	*18, 23*	
	$\|A_z\|$x10^{-4} cm^{-1}	63, 60	*64, 18*	
Glu114AlaCu_AAz	g_x	2.016,	*2.008*	70, *30*
	g_y	2.027,	*2.035*	
	g_z	2.168,	*2.219*	
	$\|A_x\|$x10^{-4} cm^{-1}	19, 23,	*26, 15*	
	$\|A_y\|$x10^{-4} cm^{-1}	30, 4	*18, 24*	
	$\|A_z\|$x10^{-4} cm^{-1}	61, 57	*60, 18*	
Glu114LeuCu_AAz	g_x	2.019,	*2.013*	90, *10*
	g_y	2.024,	*2.045*	
	g_z	2.172,	*2.216*	
	$\|A_x\|$x10^{-4} cm^{-1}	25, 23	*21, 8*	
	$\|A_y\|$x10^{-4} cm^{-1}	31, 7	*14, 18*	
	$\|A_z\|$x10^{-4} cm^{-1}	61, 54	*65, 15*	
Glu114GlnCu_AAz	g_x	2.015,	*2.007*	71, *29*
	g_y	2.027,	*2.034*	
	g_z	2.169.	*2.218*	
	$\|A_x\|$x10^{-4} cm^{-1}	15,19	*26, 15*	
	$\|A_y\|$x10^{-4} cm^{-1}	30, 6	*18, 24*	
	$\|A_z\|$x10^{-4} cm^{-1}	61, 57	*60, 18*	

Table S4: Parameters extracted from fitting of the EXAFS data of holo Cu_A samples shown in Figure S4.

Sample/Fit	F	Cu-S (Cys)			Cu-N (His)			Cu-Cu			E_0
		N	R(Å)	DW(Å^2)	N	R(Å^2)	DW(Å)	N	R(Å)	DW(Å^2)	
Glu114GlyCu$_A$Az	0.41	2	2.22	0.018	1	1.92	0.015	0.8	2.44	0.005	0.097
Glu114AlaCu$_A$Az	0.52	2	2.22	0.023	1	1.92	0.012	0.7	2.41	0.008	–0.06
Glu114LeuCu$_A$Az	0.49	2	2.19	0.02	1	1.84	0.021	0.7	2.44	0.012	3.95
Glu114GlnCu$_A$Az	0.55	2	2.16	0.014	1	1.87	0.015	0.6	2.40	0.011	7.92

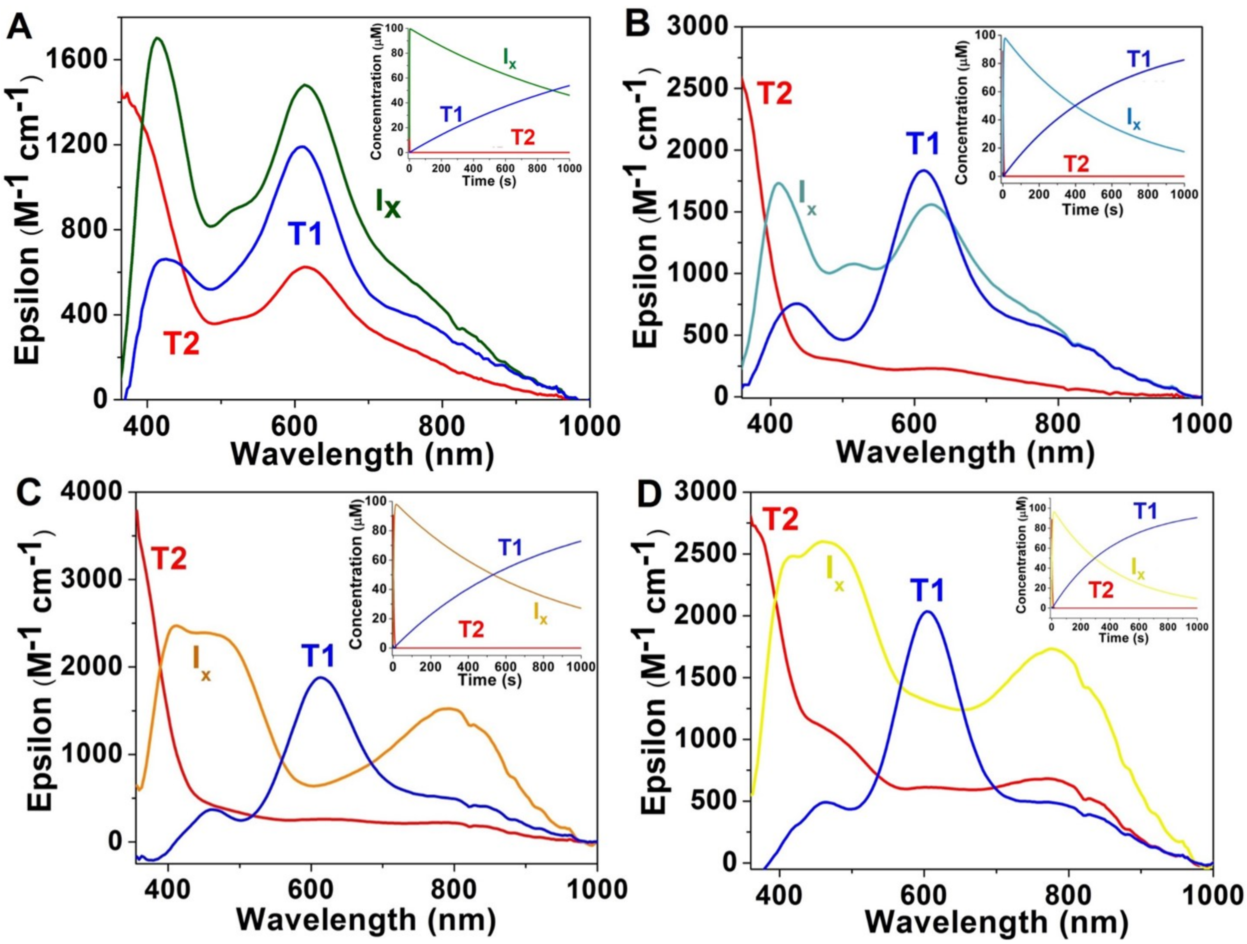

Figure S1. Specfit-derived spectra of different intermediates obtained from global analysis of the stopped-flow data of Glu114GlyCu$_A$Az (**A**), Glu114AlaCu$_A$Az (**B**), Glu114LeuCu$_A$Az (**C**), and Glu14GlnCu$_A$Az (**D**) shown in Figure 2. Concentrations of the species as a function of time are shown as insets. As $CuSO_4$ is consumed quickly, this reactant cannot be seen in the insets.

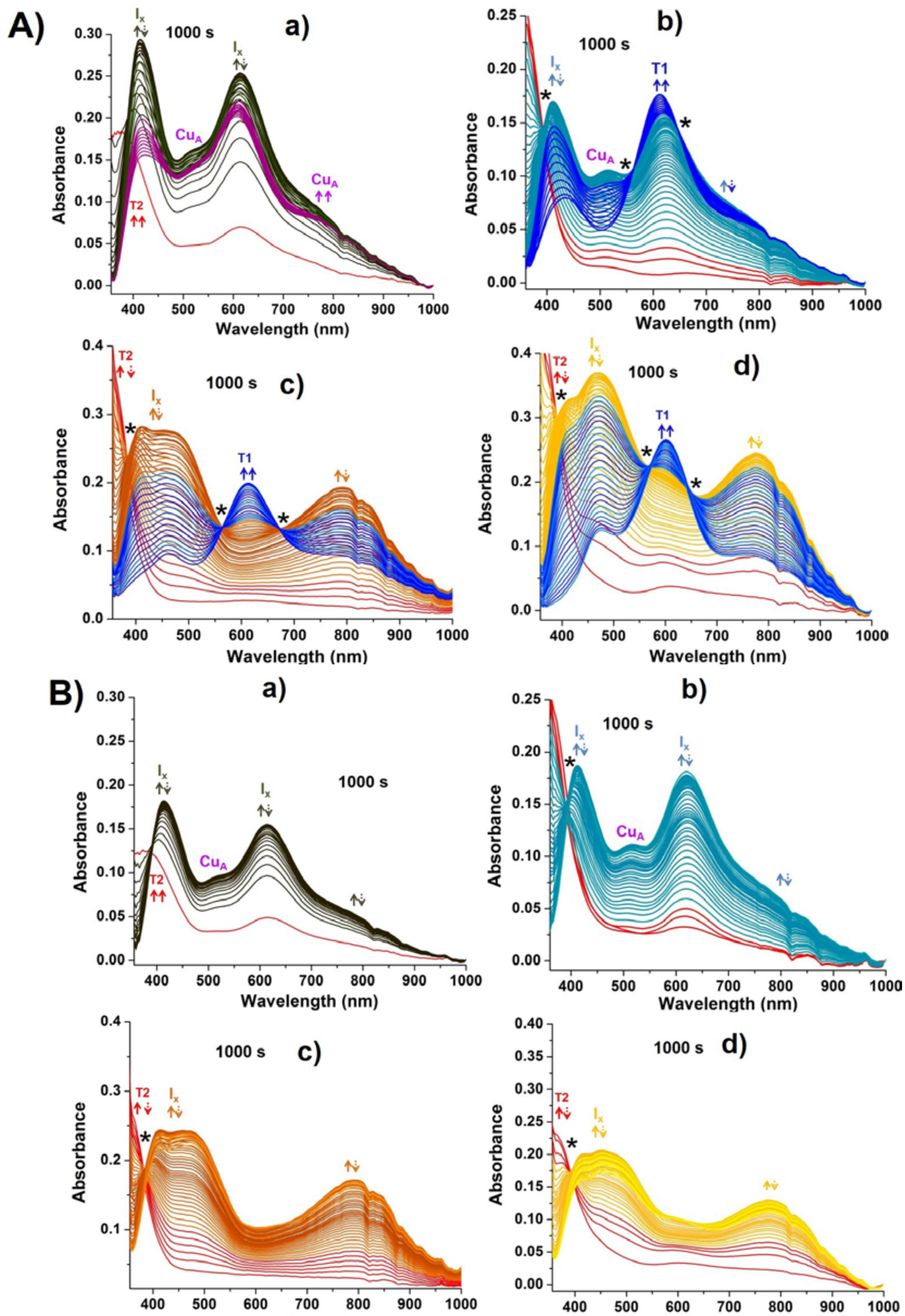

Figure S2: O_2-rich (A) and anaerobic (B) stopped flow data for all variants at pH 7. a) Glu114GlyCu_AAz, b) Glu114AlaCu_AAz, c) Glu114LeuCu_AAz, d) Glu114GlnCu_AAz.

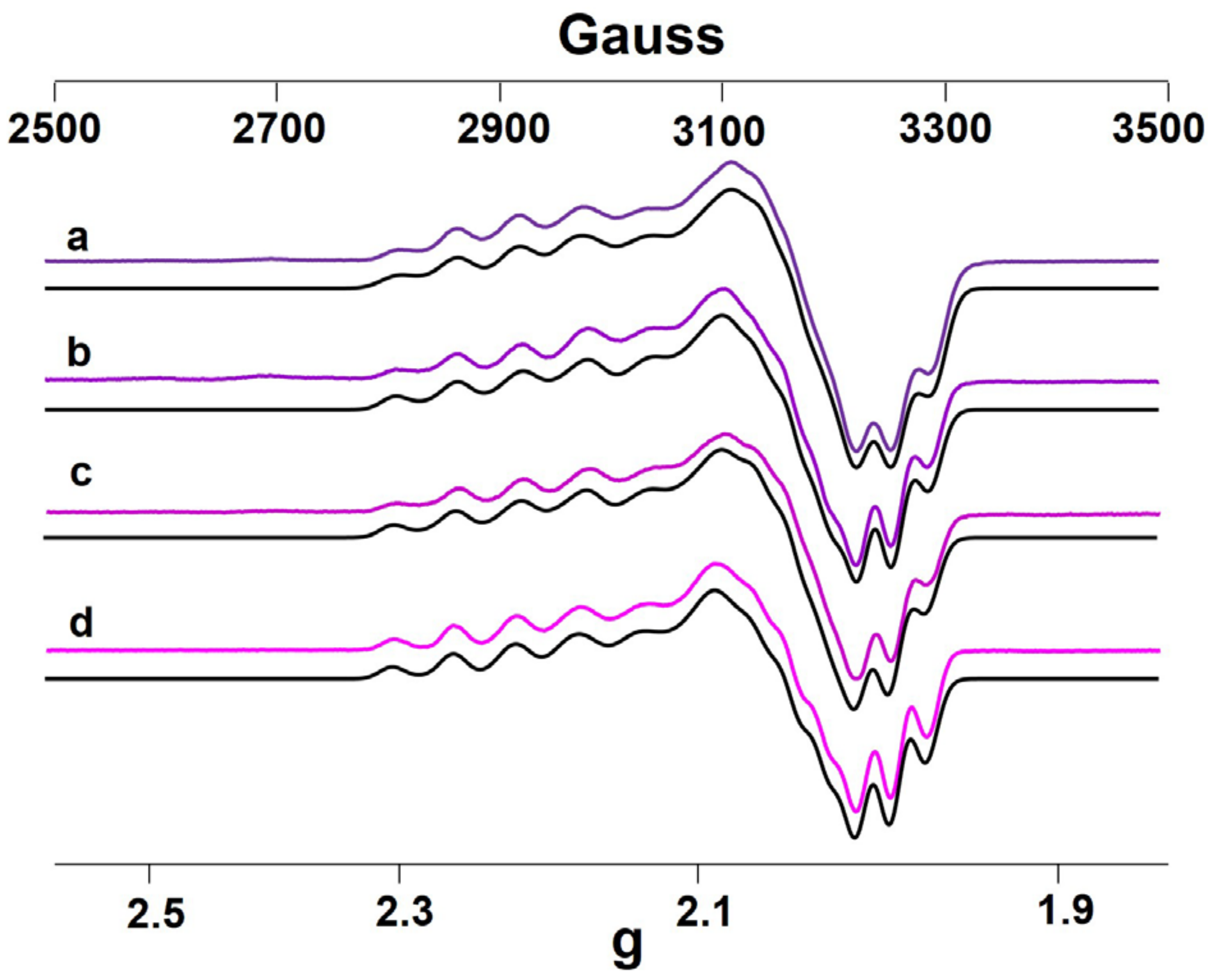

Figure. S3. EPR spectra of holo a) Glu114GlyCu$_A$Az, b) Glu114AlaCu$_A$Az, c) Glu114LeuCu$_A$Az, d) Glu114GlnCu$_A$Az prepared by mixing apo proteins with a mixture of 0.8 eq. Cu(II) and 0.8 eq. Cu(I), recorded at pH 7. Fit to the experimental data are shown as black lines. Experimental conditions: H = 9.053 GHz, T=30 K, Modulation = 4G, Microwave power = 0.2 mW.

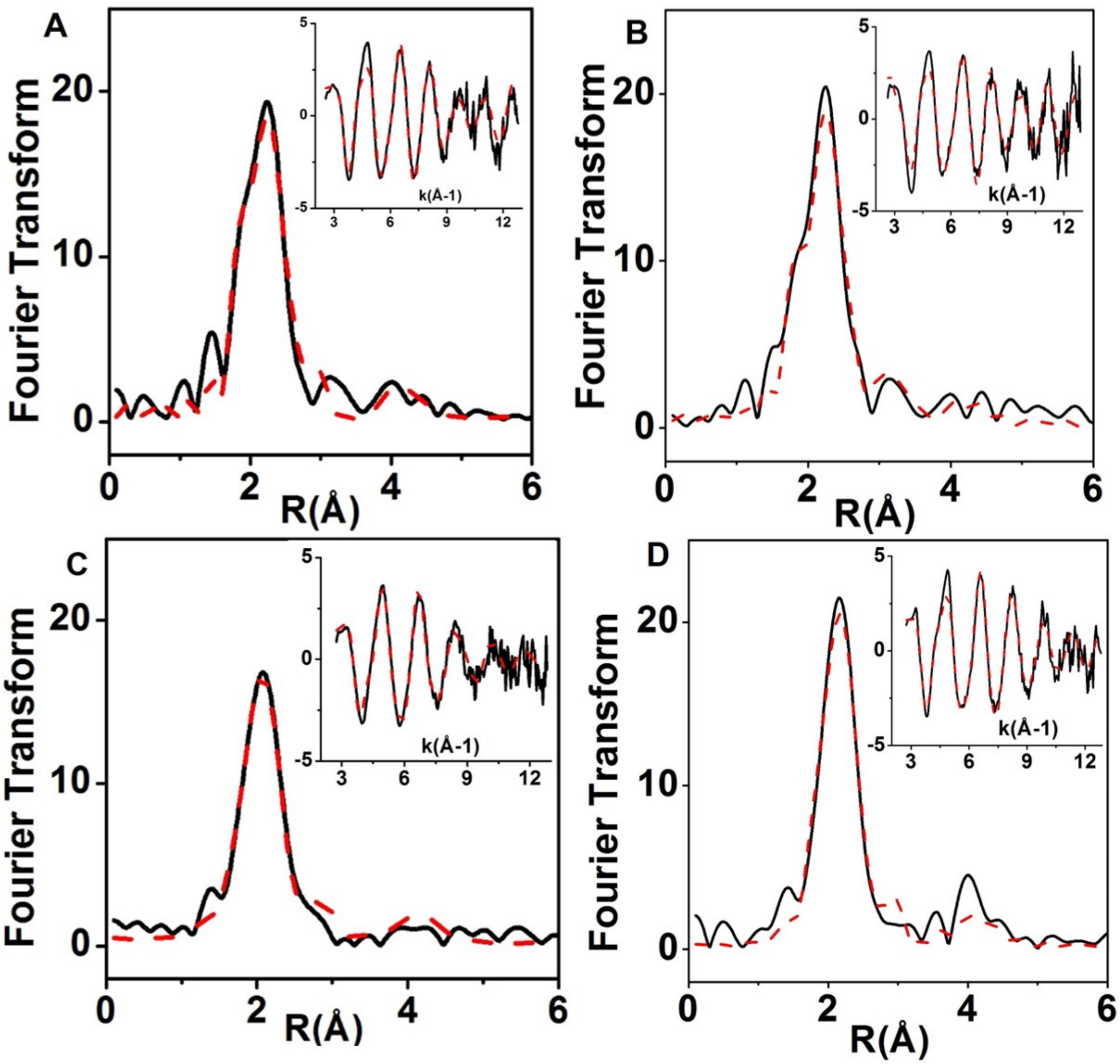

Figure S4. Fourier transform and EXAFS (inset) data of holo Cu_A samples prepared by mixing apo Glu114GlyCu_AAz (A), Glu114AlaCu_AAz (B), Glu114LeuCu_AAz (C), and Glu114GlnCu_AAz (D) with a mixture of 0.8eq Cu(II)/0.8 eq Cu(I).

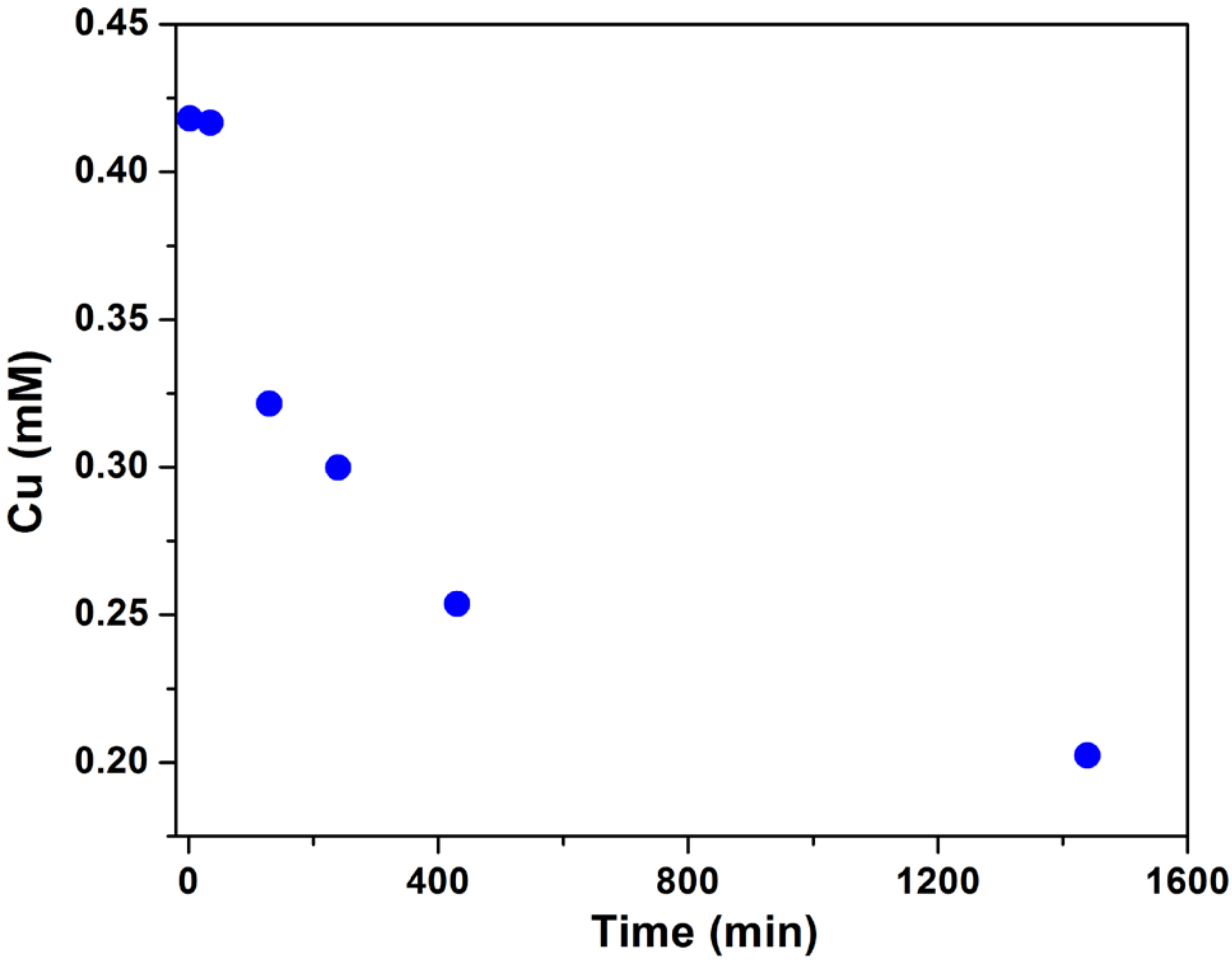

Figure S5. EPR quantification of Cu(II) present in Glu114GlyCu_AAz I_x with time. Concentration of Cu(II) is obtained from by double integration of the EPR spectra at various time points against the standard curve of $CuSO_4$. A loss of Cu(II) concentration with time suggests that spin is being lost from the system forming EPR silent Cu(I). Experimental parameters: H = 9.053 GHz, T=30 K, Modulation = 4G, Microwave power = 0.2 mW, gain = 1600.