Electronic absorption spectra of M(II)(Met121X) azurins (M = Co, Ni, Cu; X = Leu, Gly, Asp, Glu): charge-transfer energies and reduction potentials

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Abstract

Electronic absorption spectra of the Co(II) and Ni(II) derivatives of Met121X (X = Leu, Gly, Asp, Glu) azurin mutants have been measured. Coordination of carboxylate to the metal ion is indicated by LF and LMCT band shifts in the Met121Glu proteins. The relatively low reduction potentials of the Cu(II)(Met121X) (X = Asp, Glu) azurins accord with the LMCT energies of the corresponding Co(II) derivatives.

Introduction

Blue copper proteins play important roles as electron-transfer agents in photosynthesis and bacterial respiration [1]. They have also attracted attention because of their unusual properties, such as an intense charge-transfer absorption near 600 nm, a narrow hyperfine splitting in the Cu(II) EPR signal and a high reduction potential [2].

Many years ago it was suggested [3] that the unusual properties are a result of a coordination environment forced on the metal ion by the protein structure. This would be an example of a rack mechanisms, introduced by Eyring *et al.* [4] to explain the reactivity of enzyme active sites. Vallee and Williams [5] discussed a similar concept, named the entatic state, and applied it specifically to metalloenzymes, including blue copper proteins. Later, Gray and Malmström [6] used spectroscopic data to estimate the rack or entatic-state energy.

A distorted coordination environment for Cu(II) was established by crystal structure determinations of plastocyanin [7] and azurin [8]. In azurin, three strong ligands (His46, His117, Cys112) form a CuN_2S core; and, to a large extent, the electronic interactions in this core determine the spectroscopic and electron-transfer properties of blue copper [6]. The weaker interaction with Met121 apparently is much less important, since methionine is absent in the amino-acid sequence of stellacyanin [9] and one laccase [10].

Site-directed mutagenesis experiments in which Met121 in azurin was changed into other amino acids have confirmed that a methionine interaction is not a requirement for the intense blue color [11–13]. There are, however, distinct changes in the spectroscopic properties of Cu(II)(Met121X) azurins; for example, the S(Cys) → Cu(II) charge-transfer energy is slightly higher for X = Asp than for the native protein; and this ligand-to-metal charge-transfer (LMCT) transition is strongly blue-shifted in the X = Glu mutant. To examine the origin of these energy differences, we have measured the absorption spectra of four mutants (X = Leu, Gly, Asp, Glu) with Co(II) in place of Cu(II). Since the ligand-field (LF) and LMCT absorption systems are well separated in Co(II) azurin [14–16], both inner-sphere and outer-sphere interactions associated with changes at position 121 can be probed. For comparison, we also have measured the absorption spectra of the corresponding Ni(II) azurins.

Experimental

Pseudomonas aeruginosa azurin mutants were constructed by cassette mutagenesis and purified as described earlier [12].

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The Co(II) and Ni(II) derivatives of wild-type azurin were prepared as previously described [17]. The same method was used for the Gly, Asp and Glu mutants (milder conditions were employed to obtain the apoproteins, since copper binds less strongly in the mutants). Met121Leu azurin was available as the apoprotein [12]. In a typical experiment, a concentrated solution of protein was reduced with ascorbate or sodium dithionite and dialyzed twice against 50 mM thiourea in 50 mM NaOAc buffer at pH = 4.5. The apoprotein was then equilibrated with 10 mM Tris buffer pH = 7.5. Protein reconstitution was performed by adding a 5 to 10-fold excess of metal ion (a solution of cobalt(II) acetate or nickel(II) sulfate) to the apoprotein solution. Reconstitution was allowed to take place overnight at 4 °C. For X = Leu, Gly, or Asp with Ni(II), a bright yellow color developed within 30 min but the metal uptake appeared to be less than for wild-type azurin. Reconstitution of Met121Glu with Ni(II) gave a pale orange solution, while reconstitution with Co(II) resulted in a light blue solution. The proteins were purified by anion-exchange chromatography on a Mono-Q FPLC (Pharmacia) column. 10 mM Tris pH = 8.4 was used as loading buffer for Ni(II) and Co(II)(Met121Glu); DEA buffer at pH = 9.0 was used for all other proteins. The proteins were eluted with a salt gradient.

Absorption spectra were recorded using a modified Cary 14 spectrophotometer. Reduction potentials were measured by thin-layer spectroelectrochemistry [18].

Results and discussion

The LF spectra of the Co(II) derivatives of wild-type and Met121Leu azurins are strikingly similar (Table 1), thereby indicating that the S(Met)–Co(II) electronic interaction is very weak. The relatively small LF shifts in the Co(II)(Met121X) (X = Gly, Asp) proteins are probably due to slight changes in outer-sphere interactions with the metal site; however, the blue-shifted LF transitions in Co(II)(Met121Glu) azurin are more readily interpreted in terms of carboxylate coordination to the metal ion [2].

The positions of the S(Cys) → Co(II) charge-transfer bands depend strongly on the nature of the 121 residue (Table 1). By far the largest blue shifts are observed in the spectrum of the Co(II)(Met121Glu) azurin, in line with an inner-sphere carboxylate interaction. The smaller blue shift of the X = Asp LMCT system is logically attributable to an outer-sphere (solvatochromic) effect of the polar carboxylate group. Since Co(II) is reduced in the LMCT excited state, the transition energy would be expected to increase in a polar environment [19], as observed. Hydrophobic residues lower the LMCT energy, as they favor the reduced

TABLE 1. Spectroscopic data (λ (nm) ($\epsilon(M^{-1}\ cm^{-1})$) for M(II)(Met121X) azurins[a]

Co(II)(Met121)	Co(II)(Leu121)	Co(II)(Gly121)	Co(II)(Asp121)	Co(II)(Glu121)	Assignment
645sh	644sh	640 (200)	645 (560)	620 (610)	LF
638 (490)	638 (590)	620sh	625sh	603sh	LF
525sh	536 (160)	[b]	[b]	[b]	LF
522 (200)	~520sh	535 (100)	564 (240)	564sh	LF
405sh	402sh	[b]	[b]	[b]	[c]
375 (1180)	372 (1070)	358 (770)	341 (1060)	328 (1040)	LMCT
330 (3020)	327 (2940)	305 (1670)	303sh	290sh (b)	LMCT
Ni(II)(Met121)	**Ni(II)(Leu121)**	**Ni(II)(Gly121)**	**Ni(II)(Asp121)**	**Ni(II)(Glu121)**	**Assignment**
600sh	~630	~620	[b]	546sh	LF
555 (280)	~500sh	~530	[b]	499 (230)	LF
490sh	[b]	[b]	[b]	[b]	[c]
440 (3060)	418	418	414	392 (2340)	LMCT
358 (1350)	[b]	~356	~346	320 (830)	LMCT

[a]Conditions: band positions and intensities are virtually the same in 10 mM Tris pH = 8.7 and 50 mM NaP_i pH = 7.0. Molar absorbances are relative to 9140 $M^{-1}\ cm^{-1}$ (280 nm) for wild-type azurin [11]. [b]Obscured by overlapping absorptions. [c]Not assigned.

TABLE 2. Reduction potentials (mV vs. NHE) and LMCT energies (eV) for M(II)(Met121X) azurins

X	$E°$ (Cu(II)/(I))[a]	S→Cu(II)[b]	S→Ni(II)	S→Co(II)
Met	304	1.98	2.84	3.79
Leu	390	1.97	2.99	3.82
Gly	311	2.03	3.00	4.10
Asp	290	2.04	3.02	4.13
Glu	220	2.04	3.19	4.3

[a]Conditions: 100 mM KP_i; pH=7.0; 25 °C; mediator, $[Co(phen)_3](ClO_4)_3$. [b]Ref. 12.

charge on the cobalt ion in the excited state. The difference in the spectroscopic energies of the X=Asp and X=Leu proteins is 0.31 eV, which is much larger than the shift (0.07 eV) in the intense LMCT band in the corresponding Cu(II) azurins (Table 2). Calculations have shown that the S(Cys)–Cu(II) bond in the electronic ground state of blue copper is highly covalent [20], and it follows that the extent of charge transfer in the (so-called LMCT) excited state in not very large. Our spectroscopic data on analogous Cu(II) and Co(II) azurins strongly support this formulation of the S(Cys)–Cu(II) bonding.

The relatively intense absorption at 440 nm in Ni(II) azurin (Fig. 1) is attributable to S(Cys)→Ni(II) charge transfer [14–16]. This absorption blue-shifts in all four Ni(II)(Met121X) azurins (Table 1), with the largest shift for X=Glu (Fig. 1). Again, coordination of the Glu carboxylate to the metal ion is indicated. Smaller blue shifts for X=Leu, Gly, Asp suggest that the 121 environment is more polar in these proteins than for Met. The increases in the LMCT energies for X=Gly (0.16 eV) and Asp (0.18 eV) are consistent with the shifts observed for the corresponding transition in the Co(II) proteins, but the 0.15 eV blue shift of the X=Leu LMCT (relative to Met121) is hard to understand (Table 2: the LMCT energies suggest that the polarity of the 121 environment is very similar for X=Leu, Gly, Asp). One possibility is that water is present in the 121 region in these three Ni(II) mutants (and may even be coordinated to the nickel ion).

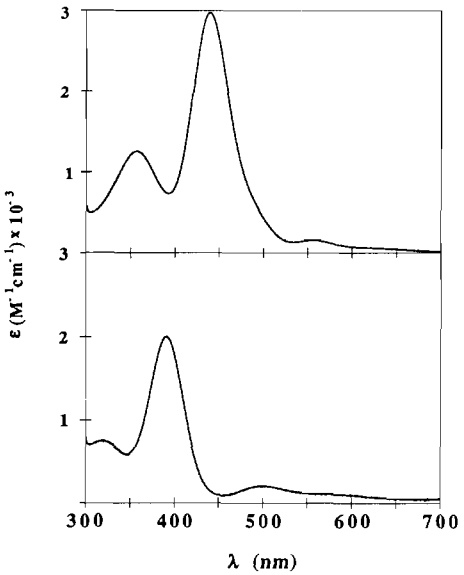

Fig. 1. Electronic absorption spectra of Ni(II)(Met121) (upper panel) and Ni(II)(Met121Glu) (lower panel) azurins at room temperature (50 mM NaP_i, pH=7.0).

The relatively weak absorption band at 555 nm in the spectrum of Ni(II)(Met121) azurin is also blue-shifted in the Glu mutant (to 499 nm; Fig. 1), thereby confirming that the Ni(II) coordination environment is perturbed. The large shift provides additional support for carboxylate coordination in this derivative.

The reduction potentials of the Cu(II)(Met121X) proteins decrease in the order Leu>Gly~Met>Asp>Glu (Table 2). If no structural rearrangement of the copper site accompanies reduction, then these potentials should correlate closely with the LMCT energies. Since formation of a S→Co(II) excited state involves extensive electron transfer, comparisons of the Co(II) LMCT energies with $E°$ (Cu(II)/Cu(I)) should be particularly revealing. Both the thermodynamic ($E°$) and the spectroscopic (LMCT) energies show clearly that a reduced metal site is destabilized in the order Met<Asp<Glu. The lack of correlation between $E°$ values and LMCT energies for X=Leu, Gly indicates that increased flexibility of the folded polypeptide chain in the mutants [12] allows some rearrangement of the coordination environment in the reduced protein. The spectroscopic energies, which would not be affected by these slight rearrangements, are more reliable indicators of the electronic stabilization of a reduced metal ion by the X group. The position of Met at the top of the order of decreasing spectroscopic reduction potentials (the order of increasing LMCT energies: Met< Leu<Gly<Asp<Glu) is consistent with the expectation that a reduced metal ion will be relatively stabilized by coupling electronically to a soft thioether sulfur atom [6]; and our finding that the Glu mutant has the lowest spectroscopic potential underscores the destabilization associated with the interaction of a hard carboxylate oxygen donor atom with an M(I) center.

Even if the presence of a methionine residue at position 121 in azurin is not a requirement for a blue site, copper ion removal is facilitated in the Met121X proteins [12]. The data presented here for the Co(II) and Ni(II) derivatives of the wild-type and mutant azurins also indicate that the metal-binding site in the mutants is more flexible than that in native azurin. Thus, methionine, although it interacts weakly with the metal, does play a structural role. This role may explain why methionine is conserved in the amino-acid sequences of all azurins and plastocyanins [21].

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References

1 O. Farver and I. Pecht, in R. Lontie (ed.), *Copper Proteins and Copper Enzymes,* CRC Press, Boca Raton, FL, 1984, p. 183.
2 R. Malkin and B. G. Malmström, *Adv. Enzymol., 33* (1970) 177.
3 L. Broman, B. G. Malmström, R. Aasa and T. Vänngård, *J. Mol. Biol., 5* (1962) 301.
4 H. Eyring, R. Lumry and J. D. Spikes, in W. D. McElroy and B. Glass (eds.), *Mechanism of Enzyme Action,* Johns Hopkins Press, Baltimore, MD, 1956, p. 123.
5 B. L. Vallee and R. J. P. Williams, *Proc. Natl. Acad. Sci. U.S.A., 59* (1968) 498.
6 H. B. Gray and B. G. Malmström, *Comments Inorg. Chem., 2* (1983) 203.
7 J. M. Guss and H. C. Freeman, *J. Mol. Biol., 169* (1983) 521.
8 E. T. Adman and L. H. Jensen, *Isr. J. Chem., 21* (1981) 8.
9 C. Bergman, E. K. Gandvik, P. O. Nyman and L. Strid, *Biochim. Biophys. Res. Commun., 77* (1977) 1052.
10 U. A. Germann, G. Müller, P. E. Hunziker and K. Lerch, *J. Biol. Chem., 263* (1988) 885.
11 B. G. Karlsson, R. Aasa, B. G. Malmström and L. G. Lundberg, *FEBS Lett., 253* (1989) 99.
12 B. G. Karlsson, M. Nordling, T. Pascher, L. C. Tsai, L. Sjölin and L. G. Lundberg, *Protein Eng., 4* (1991) 343.
13 T. K. Chang, S. A. Iverson, C. G. Rodrigues, C. N. Kiser, A. Y. C. Lew, J. P. Germanas and J. H. Richards, *Proc. Natl. Acad. Sci. U.S.A., 88* (1991) 1325.
14 H. B. Gray and E. I. Solomon, in T. G. Spiro (ed.), *Copper Proteins,* Wiley, New York, 1981, p. 2.
15 D. R. McMillin, R. C. Rosenberg and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A., 71* (1974) 4760.
16 D. L. Tennent and D. R. McMillin, *J. Am. Chem. Soc., 101* (1979) 2307.
17 J. A. Blaszak, D. R. McMillin, A. T. Thornton and D. L. Tennent, *J. Biol. Chem., 258* (1983) 9886.
18 W. R. Ellis, *Ph. D. Thesis,* California Institute of Technology, 1986.
19 A. B. P. Lever, *Inorganic Electronic Spectroscopy,* Elsevier, Amsterdam, 2nd edn., 1984.
20 A. A. Gewirth and E. I. Solomon, *J. Am. Chem. Soc., 110* (1988) 3811.
21 L. Rydén, in R. Lontie (ed.), *Copper Proteins and Copper Enzymes,* CRC Press, Boca Raton, FL, 1984, p. 157.