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CHEMICAL MODIFICATION OF METHIONINE RESIDUES IN AZURIN

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Summary. Azurin from Pseudomonas aeruginosa has been treated with bromoacetate at low pH to alkylate methionine residues. Two classes of methionine side chains are observed as a result of these reactions - four of the six methionines are reactive at pH 4, whereas all six are reactive at pH 3.2. The product containing four alkylated methionines maintains a significant portion of the blue color and spectroscopic characteristics of the native protein. The product which has been fully modified at the methionine residues, on the other hand, has lost all blue color and appears to be largely in a random coil form.

The recent report concerning the three-dimensional structure analysis of azurin from Pseudomonas aeruginosa has suggested that a methionine residue is spatially close to the copper atom in this protein, and perhaps its sulfur atom is one of the ligand groups (1). In addition a cysteine and two histidine side chains, as has been suggested from previous evidence (2-6), may contribute ligand groups to the metal. A similar coordination sphere for the Cu atom in another blue protein, plastocyanin, has been postulated from x-ray diffraction data (7). As yet, however, the importance of the specific groups within the protein in the copper binding and the electron transfer activity of azurin has not been totally defined. In an effort to further delineate the importance of some of these groups on the structure and reactivity of the protein, we have undertaken a series of site-specific chemical modifications to the azurin molecule. This report constitutes a preliminary account of our studies that focus on the modification of methionine side chains by bromoacetate.

MATERIALS AND METHODS. Azurin was prepared from isolated cells of Pseudomonas aeruginosa as previously described (8). The apoprotein was prepared by dialyzing a solution of azurin against a cyanide solution at pH 10 (9). For composition analysis, protein samples were hydrolyzed in 6N HCl, dried, and resuspended in pH 2.2 citrate buffer before being applied to a Beckman 119B amino acid analyzer. The extent of modification of methionine residues was analyzed

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by subjecting the modified protein to peroxidation (10) and measuring the amount of unmodified methionine as methionine sulfone (10,11). Total cysteine was quantitatively analyzed either as cysteic acid following peroxidation (10) or as the sulfocysteine derivative following the method of Inglis and Liu (12).

Carboxymethylation of the protein was carried out under different conditions. Both bromoacetate and iodoacetamide were used as alkylating agents, the concentrations of reagent and time of reaction were varied, the pH of the medium was varied, and both holo- and apoprotein were modified. In a typical reaction, the protein was dissolved in a buffer solution at the appropriate pH, a small volume of a concentrated solution of the modifying reagent was added to give the desired concentration, and the pH was readjusted if necessary. The reaction mixture was left in the dark for the desired length of time at room temperature (23°C), excess reagent was removed either by gel filtration or dialysis, and the modified protein solution was lyophilized and stored at -20°C.

RESULTS. Although both bromoacetate and iodoacetamide have been used as alkylating agents, most of the reactions performed thus far have been with bromoacetate, and these form the basis of the results reported here. Qualitatively similar results have been obtained with iodoacetamide, but the reaction appears to be slower and less complete.

Ambler and Brown have previously reported that the cysteine residue at position 112, which is thought to be bound to the copper atom (2,3), is resistant to alkylation, even in the denatured apoprotein (2). We have confirmed the lack of reactivity of this cysteine toward bromoacetate (0.16M) at pH 7 and 8 for 24 hr, conditions under which the sulfhydryl group would be expected to be reactive. Furthermore, there appears to be little if any reaction of the histidine side chains toward bromoacetate at these higher pH values (Table I). On the other hand, if the alkylation reaction is carried out under the same conditions using the apoprotein, the amount of free histidine recovered is markedly diminished. In addition, only a trace of methionine sulfone is seen following peroxidation of the sample; this suggests that essentially all of the methionine is carboxymethylated in the apoprotein at pH 7.

In spite of the fact that alkylation does not seem to occur at cysteine and histidine in the holoprotein, the reactions directed specifically toward methionine side chains have been carried out at low pH to ensure lack of

TABLE I

Amino Acid Composition of Azurin Samples Following Modification with Bromoacetate[a]

Amino Acid	Residues per Molecule of Protein				
	Azurin unmodified	Azurin reacted with BrAc, pH7	Apoazurin reacted with BrAc, pH7	Azurin reacted with BrAc, pH3.2	Azurin reacted with BrAc, pH4.0
Aspartic Acid	16.3	17.2	16.9	15.7	16.6
Threonine	8.8	9.4	7.7	8.9	9.2
Serine	7.9	8.8	8.5	8.2	9.1
Glutamic Acid	10.3	10.5	11.2	10.4	10.1
Proline	4.1	4.2	3.2	3.8	4.0
Glycine	10.9	10.9	10.6	10.8	10.9
Alanine	8.0	7.9	7.3	8.6	8.4
Cysteine	3.1[b]	2.9[b]	2.9[c]	2.3[b]	2.5[b]
Valine	10.1	9.9	9.2	9.6	9.7
Methionine	5.9	3.5	1.6	1.7	3.0
Methionine Sulfone[d]	(5.8)	2.9	trace	0.4	1.9
Isoleucine	4.3	4.0	4.1	4.3	4.2
Leucine	9.6	9.9	6.7	9.5	9.6
Tyrosine	2.0	2.0	2.2	2.2	2.1
Phenylalanine	5.8	5.8	6.2	5.8	5.7
Histidine	3.9	3.7	1.6	3.9	3.7
Lysine	10.6	11.0	9.0	10.7	10.5
Arginine	1.2	1.1	1.2	1.3	1.2

[a] 24 hr hydrolysis. No corrections were made for losses of serine and threonine.

[b] Measured as the sulfocysteine derivative.

[c] Measured as the cysteic acid derivative following peroxidation.

[d] Measured on an equivalent sample after peroxidation.

reactivity of the imidazole and sulfhydryl group. The standard set of reaction conditions consists of a 0.1M sodium formate buffer at pH 3.2 or 4.0, 0.16M bromoacetate, for 24 hr at room temperature.

A clear distinction can be made between the results of the alkylation reactions carried out at pH 3.2 and 4.0. At pH 4.0 after 24 hr the solution of copper protein retains its blue color, whereas at pH 3.2, the blue color gradually fades until after about 12 hr it is no longer apparent. Addition of copper(II) salts or potassium ferricyanide fails to regenerate any further blue color, so it seems that neither reduction nor reversible removal of the

Cu(II) atom is responsible for the loss of color. Solutions of azurin left at pH 3.2 for 24 hr in the absence of bromoacetate do not show any comparable color loss.

Products of the azurin alkylation reaction at the two pH values were hydrolyzed and subjected to amino acid analysis (Table I). It is clear that there is a significant difference in the amount of methionine that has reacted with bromoacetate at the two different pH values. We have also consistently observed a decline in the levels of analyzable cysteine with the low pH alkylations, the losses being less severe at pH 4.0 than at pH 3.2, where decoloration occurs. In neither case, however, does the loss of cysteine amount to the equivalent of one residue per molecule. No losses of histidine are observed at either pH value.

The analyses given in Table I represent the average values of unfractionated mixtures of the alkylated product. Analytical gel electrophoresis (pH 8.3) and isoelectric focusing of the product mixtures show the formation of several minor components. This heterogeneity, as might be expected from the amino acid analyses, is most severe for the apoazurin samples alkylated at pH 7.

Preliminary spectral analyses of the modified protein samples show that for the protein alkylated at pH 3.2, the absorbance at 625 nm is virtually nonexistent, while the UV region of the spectrum is devoid of most of the fine structure characteristic of both apo- and holoazurin. In spite of the fact that the protein alkylated at pH 4 still exhibits the blue color characteristic of azurin, the ratio of the absorbance at 625 nm to that at 280 nm has decreased by about 30%. Whether this represents a decrease in the absorbance at 625 nm or an increase in the absorbance at 280 nm (or a combination of the two) is not clear at this time. Some of the fine structure in the UV region is lost; in particular the sharp band at 290 nm, though still present, is less well defined. The far UV region of the CD spectrum of this sample shows a

decrease in the negative extremum at 220 nm seen in native azurin (13), but in the sample alkylated at pH 3.2, this region of the spectrum suggests a totally random coil structure.

DISCUSSION. Modification of all six of the methionine residues of azurin leads to a product which bears little spectral resemblance to the native protein. Neither the absorbance at 625 nm, which is characteristic of the copper(II) protein, nor the 220 nm negative trough in the CD spectrum, which is related to the secondary structure of the molecule, is present. On the other hand, by changing the pH of the alkylation reaction, a product is obtained in which an average of four of the six methionine side chains is modified. This product retains to a large degree the spectral characteristics of the native protein, and at least qualitatively maintains its blue color.

The reaction between bromoacetate and accessible methionine residues in proteins is independent of pH (14). Thus it would appear that the increased reactivity of two methionine residues as the pH is decreased from 4.0 to 3.2 results from an increased accessibility of these side chains toward the reagent. Spectral titrations of azurin suggest that the absorbance at 625 nm of the protein is maintained to very low pH, and is essentially unchanged at pH 3.2 (3,15). On the other hand, the increased reactivity of the two extra methionine side chains appears to correlate with our observations that the fluorescence emission at 308 nm begins to decrease below pH 4 and is essentially nonexistent at pH 3 (15). It seems, therefore, that there is a conformational change in the protein as the pH is decreased below 4 which does not affect the Cu(II) chromophore directly but does allow bromoacetate to react with two previously unreactive thioether groups. As a result of the modification of one or both of these groups, the integrity of the Cu(II) environment is destroyed, and the characteristic blue color is lost. We cannot say at this point whether the Cu(II) atom is totally displaced from the protein or whether it remains bound, but in a nonspecific way.

It is tempting to try to correlate these results with the structural information derived from the x-ray diffraction analysis of azurin at 3Å resolution (1). Three of the methionine residues (#109, 64 and 56) appear to be at or near the surface of the molecule and not near the Cu atom. Another (#44) may be near the surface but may help "shield" the Cu atom from solvent. Methionine 121 is postulated to be very near the Cu atom, and it is suggested it may provide a ligand to the metal. The orientation of methionine 13 is not given, although Adman et al. (1) give no evidence of its reactivity toward Pt in the heavy atom derivatives. Thus, methionines 109, 64, 56 and either 13 or 44 might be expected to react with bromoacetate at pH 4, whereas methionine 121 and perhaps 44 (or 13) would be expected to be unreactive. Lowering the pH to 3.2 appears to affect the environment of the tryptophan at position 48 and may well also change the reactivity of the nearby methionine 44. If Met 44 is indeed involved in "shielding" the Cu environment, modification of this group might well render the Cu binding site area (including Met 121) accessible to solvent. We are currently attempting to isolate and purify the principal product that results from the bromoacetate reaction at pH 4 so that we can determine which methionines have been modified. It should then be possible to test these hypotheses.

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