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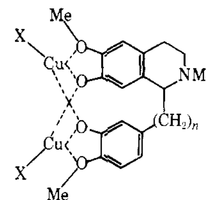

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Isomerization of (*Z*)-Arenediazo Thioethers on Aldolase and Model Compounds

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Abstract: Arenediazo derivatives of *N*-acetylcysteine, having different substituent groups in para position, have been prepared. The decomposition and isomerization kinetics of the *Z* isomers of these chromophores have been investigated in water solution. The nature of the para substituent group in the aryl moiety affects activation parameters for both reactions. Electron-withdrawing groups stabilize the *Z* isomer giving slower reaction rates for both thermal conversion to the *E* isomer and decomposition; electron-donating groups bring about rate enhancements. A linear enthalpy-entropy relationship and a fair linearity in the Hammett plot support the existence of a unique isomerization mechanism. The *p*-nitro- and *p*-carboxy-substituted benzenediazo thioethers have been formed on aldolase. In both cases the protein moiety lowers the activation energy of the isomerization. With the *p*-carboxybenzeneazoaldolase a first-order decay of the *Z* isomer is observed when the number of extrinsic chromophores is one per protein chain; when the number is two (in this case they are known to be located at Cys 237 and Cys 287), the time decay curve can be produced by the sum of two different exponential components, one of which is similar to that observed when only one extrinsic chromophore is present per chain. The two processes have been rationalized tentatively with the different location of the two chromophores on the enzyme.

Diazo thioether chromophores are formed by coupling diazonium salts with proteins having free sulfhydryl groups.[2a] They are fairly stable in neutral solutions, and show photosensitivity due to *E*/*Z* isomerization.[2b] A biologically active azoaldolase has been obtained, in which the modified cysteine residues are Cys 237 and Cys 287.[3] This provides the possibility of using the diazo thioethers as optical probes[4] for selected regions of a protein.

The properties of these chromophores are markedly influenced by the substituent in the para position of the aryl moiety. In order to choose the most suitable group for a protein probe, as regards chemical stability, lifetime of the metastable *Z* state, and sensitivity to the environment, we have studied a series of compounds, prepared by reaction of *N*-acetyl-L-cysteine with the aromatic diazonium salts, bearing the appropriate substituent in the para position of the aryl moiety (Scheme I).

The *p*-NO_2 and *p*-COO^- substituted benzenediazo thioethers have been investigated covalently linked to aldolase. Indications of interaction with the protein have been found. The properties of the *p*-carboxylate benzenediazo chromophore suggest its possible application as an optical probe for local protein conformation.

Experimental Section

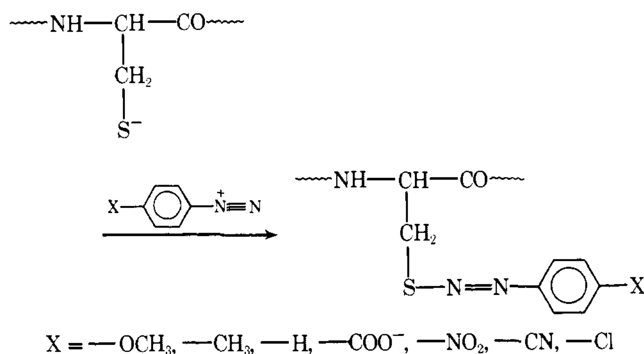
Cysteine Derivatives. Benzenediazo-substituted cysteine derivatives were prepared as already reported[5] by coupling the appropriate diazonium salt with a water solution of *N*-acetyl-L-cysteine (Serva)

Table I. Physical and Analytical Data

Substituent	λ_{max}, nm[a] (log ϵ)	Calcd, % C	Calcd, % H	Calcd, % N	Calcd, % S	Found, % C	Found, % H	Found, % N	Found, % S
p-OCH_3	340 (4.20)	48.47	5.08	14.13	10.78	48.64	5.07	13.82	11.10
p-CH_3	330 (4.20)	51.05	5.35	14.89	11.36	51.73	5.36	14.69	11.73
H	328 (4.12)	49.42	4.90	15.72	12.00	50.01	4.95	15.35	12.55
p-COO^- [b]	329 (4.19)	46.30	4.21	13.50	10.30	46.61	4.26	12.90	9.92
p-Cl[c]	329 (4.09)	43.78	4.01	13.93	10.62	44.37	4.26	13.26	10.26
p-CN	333 (4.12)	49.30	4.14	19.17	10.97	48.56	4.36	18.10	11.40
p-NO_2	345 (4.12)	42.29	3.87	17.95	10.26	42.84	3.78	17.63	10.70

[a] Solvent: 10 mM phosphate buffer, pH 7.2. [b] Reference 4. [c] Chemical analysis: Cl, calcd 11.76; found 12.06.

Scheme I



saturated with ethyl acetate. The compound is extracted in acid conditions with purified ethyl acetate and crystallized, in concentrated solution, by dropwise addition of petroleum ether under red light. To avoid formation of the thioether, the amine needed to be carefully purified, especially in the case of the p-chloroaniline; a convenient additional procedure was to extract the solution of the amine hydrochloride with ethyl acetate before diazotization.

Although the synthesis initially produced the Z isomer of the diazo thioether,[5] after the purification procedure all the compounds were obtained as the E isomers except in the case of the p-NO_2 derivative, where the compound could be crystallized practically in the Z form. The sample we used had λ_{max} 310 nm (A = 0.39, cell 1 cm, c 0.0177 g/L). We have not determined the actual content in the Z isomer, but estimated it to be not less than 80%. All the compounds proved to be chemically stable when stored in the crystalline state (at least for 1 month). Physical and analytical data are given in Table I. The structure of the compounds was also verified by IR analysis.[6]

Azoaldolase. Azoaldolases were prepared in 50 mM phosphate buffer, pH 6, at 0 °C, with a molar ratio of diazonium salt/protein of 20/1. The reaction was started by the addition of the diazonium salt to the protein solution, and stopped by gel filtration on a Sephadex G 25 column equilibrated in 10 mM phosphate buffer, pH 7.2. All the procedures were carried out under dim red light.

When the reaction was stopped immediately after addition of the reactants, the azoaldolase was found to be homogeneous with one diazo thioether group per protein chain. When the reaction was stopped 1 h after the addition of the reactants the azoaldolase had two diazo thioether groups per protein chain.

The modified proteins retained the biological activity of the native enzyme:[2a] turnover number = 3600 mol of fructose 1,6-bisphosphate cleaved per min per mol of enzyme at 37 °C, K_M = 33 μM.

Kinetic Measurements. Solutions of the compounds were prepared in 10 mM phosphate buffer, pH 7.2, under dim red light and immediately used for measurements.

The isomerization that was followed was the thermal conversion from the metastable Z form to the stable E form. With the exception of the p-NO_2 substituted derivative, a suitable amount of Z isomers of the other compounds was generated by light irradiation of the E isomers in aqueous solutions (yield 30–50%); for the low molecular weight compounds the source was a Braun F 700 electronic flash lamp filtered by a Filtraflex K2 broad band interference filter; the protein was irradiated as reported.[2b] The isomerization rates were measured by following the absorbance recovery in the dark at the λ_{max} of the π,π^* band of the E isomer. The temperature ranges were from 5 to 20 °C for p-OCH_3 and p-CH_3 substituted compounds and for the unsubstituted derivative, and from 20 to 35 °C for the p-Cl, p-COO^-, p-CN, and p-NO_2 substituted model compounds and for the azoaldolases. In these conditions decomposition was not detectable in the time scale of the isomerization measurements.

The decomposition reactions were studied spectrophotometrically by starting with solutions of pure E isomers and following the decrease of absorbance at the λ_{max} of the π,π^* band. The temperature range was usually from 20 to 50 °C.

The rate constants were calculated by least-squares analysis of the decay curve. Activation parameters were calculated with the least-squares analysis of an Arrhenius plot; the estimated standard deviations for activation energies were within ±5%.

Results

Thermal Isomerization of the Low Molecular Weight Models. The arenediazo thioethers in the E form show a strong absorption at ~330 nm due to a π,π^* transition. In the Z form, the band is displaced in the UV region; for example, λ_{max} is shifted 68 nm ($\Delta\epsilon$ = 13 600 at 330 nm) in the case of the p-COO^- substituted derivative. The irradiation of a solution of the compounds causes a decrease in the absorbance in the band of the E isomers. The original value is fully restored in the dark, if the irradiation is carried out with visible light (450 nm) for a short period of time. This inverse photochromism can be used as a sensitive isomerization detector. In Table II are summarized the results obtained with this method.

The values of the first-order rate constant show that the substituent affects the reaction. The Hammett plot at 15 °C gives a fairly straight line (correlation coefficient −0.981), the ρ value being − 2.7 (Figure 1, dashed line). Both activation energies and frequency factors appear to increase with the substituent constant σ of the group in para position.

A linear relationship (correlation factor −0.990) has been found to apply to the enthalpy and entropy changes, indicating the applicability of the "isokinetic temperature" concept (isokinetic point = 366 K).[7] Isokinetic relationship and linearity in the Hammett plot suggest that the reaction mechanism will not change by changing the group in para position to the benzene ring.[7]

Decomposition of the Low Molecular Weight Models. The investigated compounds proved to be stable to a different extent when neutral aqueous solutions were kept in the dark. As reported in Table III, the substituent produces a clear effect on the kinetic parameters of the decomposition. The Hammett plot at 35 °C gives a straight line with negative slope (Figure 1, full line, ρ = −3.9), as found for the isomerization reaction. These data are consistent with a mechanism based on the equilibration of the Z isomer with the ion pair, diazonium cation–sulfhydrylate anion, the rate-limiting step being the thermal $E \rightarrow Z$ conversion, as previously suggested for the p-carboxylate derivative.[5] This interpretation is in agreement with the findings of Brokken-Zijp and Bogaert[8] on decomposition of arenediazo *tert*-butyl thioethers and with the con-

Table II. Activation Parameters for $Z \rightarrow E$ Thermal Isomerization of Para-Substituted Benzenediazo Thioethers of *N*-Acetylcysteine in 10 mM Phosphate Buffer, pH 7.2

Substituent	E_a, kcal/mol	Log A	$\Delta S^{\ddagger}$,[a] eu	$\Delta H^{\ddagger}$,[a] kcal/mol	$\Delta F^{\ddagger}$,[a] kcal/mol	$k \times 10^3$,[a] s^{-1}
p-OCH_3	17.5	11.2	−9.2	16.9	19.5	8.6
p-CH_3	18.8	12.1	−5.1	18.2	19.7	7.8
H	21.7	14.1	+4.1	21.1	19.9	5.4
p-COO^-[b]	19.8	12.2	−4.6	19.2	20.5	1.4[c]
p-Cl	25.2	16.2	+13.7	24.6	20.6	1.21[c]
p-CN	27.0	15.9	+12.3	26.4	22.8	0.026[c]
p-NO_2	34.1	21.2	+36.5	33.6	23.1	0.022[c]

[a] Values at 15 °C. [b] From ref 4. [c] Extrapolated values.

Table III. Activation Parameters for Decomposition of Para-Substituted Benzenediazo Thioethers of *N*-Acetylcysteine in 10 mM Phosphate Buffer, pH 7.2

Substituent	E_a, kcal/mol	Log A	$\Delta S^{\ddagger}$,[a] eu	$\Delta H^{\ddagger}$,[a] kcal/mol	$\Delta F^{\ddagger}$,[a] kcal/mol	$k \times 10^4$ [a] s^{-1}
p-OCH_3	18.5	10.3	−13.5	17.9	22.0	15.0
p-CH_3	23.1	13.2	−0.1	22.5	22.5	6.25
H	22.9	12.5	−3.4	22.3	23.3	1.35

[a] Values at 35 °C.

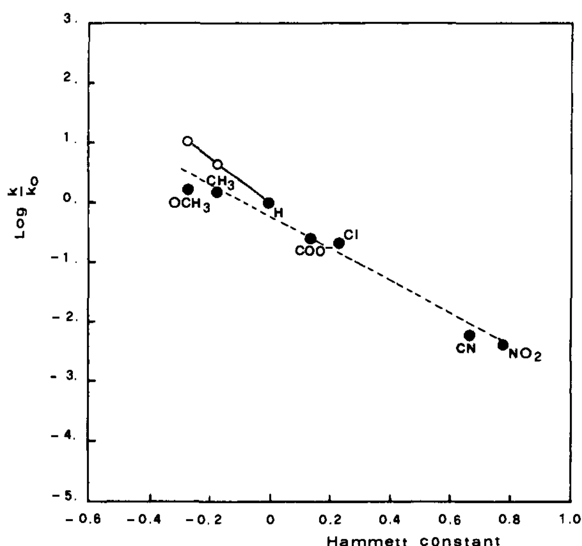


Figure 1. Hammett plots of para-substituted benzenediazo thioethers of *N*-acetylcysteine in 10 mM phosphate buffer, pH 7.2: ------- ● -------, $Z \rightarrow E$ thermal isomerization (15 °C); — O —, decomposition (35 °C).

clusions of van Zwet and Koyman[9] on similar compounds.[10]

The substituent effect found in the decomposition of diazo thioethers confirms the observation of Yamada et al.[12] that a more efficient stabilization of the ion pair is brought about by the electron-donating substituents than by the electron-withdrawing ones. This explains the fair stability of the derivatives of *N*-acetylcysteine substituted with electron acceptor groups in the temperature range 20–35 °C.

Thermal Isomerization in Azoaldolase. The p-COO^- and p-NO_2 substituted benzenediazo thioethers have been investigated when bound to aldolase. The features of their absorption spectra in the near-UV region are retained. On the contrary the protein moiety affects the energy barriers and the entropy terms of the thermal $Z \rightarrow E$ conversion, which have been found lower than in the models (Table IV). This effect is more marked in the case of the p-NO_2 substituted diazo thioether.

The azoaldolase obtained with the p-carboxy-substituted diazonium salt shows an interesting peculiarity. When in the macromolecule two cysteinyl residues per peptide chain have been modified, the time decay of the Z isomers to the E form can be satisfactorily analyzed as the sum of two exponential laws. The activation parameters associated with one of these laws are very similar (1) to those observed when only one diazo thioether chromophore has been formed per chain; (2) to the ones of the low molecular weight model (Table II). The p-nitro-benzeneazoaldolase produces a time decay curve of the Z isomers well described by a single exponential law, even when two azo chromophores per chain have been formed.

Discussion

Optical probes for proteins have been proposed for collecting information from selected regions of the macromolecule in solution. Optical properties[13] and photochromism[4] of azobenzenes have been employed. The interest of the diazo thioethers in azo proteins has been underestimated, because they were thought to be unstable in water solution.[14] Indeed in the investigated series of para-substituted benzenediazo thioethers of *N*-acetylcysteine, we have found that not only the compounds substituted with electron donors are unstable in water solution, but also the unsubstituted compounds. However the derivatives substituted with electron-withdrawing groups are fairly stable and show the longest lifetime of the Z isomers thus being more useful for practical applications.

Arene diazo thioethers undergo by light irradiation a major but revertible change of geometry, which is subject to steric constraints when the chromophores are bound to a protein. The comparison of the parameters of the thermal isomerization of the chromophores when free in solution or bound to the protein can be used to describe the protein microenvironments in which they are located.

In aqueous solution the low molecular weight models exhibit strong influence of the group in the para position on the isomerization thermodynamic parameters. The substituent effect on the rate constants confirms a trend previously observed in polar, nonaqueous solvents,[8] although in water more marked differences between donor and acceptor substituents are found. The data indicate that a unique mechanism is op-

Table IV. Activation Parameters for $Z \rightarrow E$ Isomerization of Para-Substituted Benzeneazoaldolases in 10 mM Phosphate Buffer, pH 7.2

Substituent	E_a, kcal/mol	Log A	$\Delta S^{\ddagger}$,[a] eu	$\Delta H^{\ddagger}$,[a] kcal/mol	$\Delta F^{\ddagger}$,[a] kcal/mol	$k \times 10^3$ [a,d] s^{-1}
p-COO^-	16.3[b]	9.5	−17.0	15.7	20.5	1.37
	16.2[c]	9.6	−16.4	15.6	20.3	2.00
	17.9[c]	9.8	−15.6	17.3	21.7	0.19
p-NO_2[e]	23.9	14.0	+3.6	23.3	22.3	0.07

[a] Values at 15 °C. [b] Azoaldolase with one chromophore per chain. [c] Azoaldolase with two chromophores per chain: values obtained by analysis of the decay curves of the Z isomer as sum of two exponential components, using a nonlinear least-squares computer program. The obtained Arrenhius plots have correlation coefficients better than −0.995. [d] Extrapolated values. [e] Azoaldolase with two chromophores per chain.

erating in all the compounds. The planar inversion mechanism, which is generally assumed to be present in monosubstituted azobenzenes,[16-19] has been proposed for the thermal isomerization in these compounds, on the basis of the low energy barriers and of the relatively small solvent effects which have been observed.[8,15] Contributions of polar structures to the inversion transition state have also been proposed.[8] The results we had in water solution do not correspond exactly with this model. In fact higher activation barriers were found in the derivatives substituted with electron acceptor groups, in which on the contrary the formation of the inversion transition state should have been facilitated.[21] The dependence of the activation parameters on the substituent and the negative ρ value in the Hammett plot could instead be consistent with a rotational mechanism in which the transition state was stabilized by dipolar resonance structures, as previously suggested for para-donor/para′-acceptor substituted azobenzenes[22] and for various N-phenylimines with electron donor substituents in para position.[21] This supposition is also supported by our previous observation that the solvent polarity had a marked effect on the $Z \rightarrow E$ thermal isomerization rates in p-carboxylate benzenediazo-N-acetyl-L-cysteine.[5]

When the diazo thioether groups are covalently bound to aldolase, the major change in the environment changes the activation parameters for the thermal isomerization. In the p-NO_2 azo derivative of the protein these changes are so relevant that the possibility of a novel isomerization mechanism must be taken into account. However, the mechanism operating in the model can be retained in the case of the p-carboxylate derivative, where the small changes in the activation parameters are reasonably explained by the steric constraints of the protein moiety on the azo chromophores, so as to destabilize the Z isomers, and by a better solvation of the transition state.

The p-carboxylate benzenediazo thioether appears sensitive to the protein microenvironment if the isomerization rates are considered. The presence of double exponential decays makes this chromophore a convenient optical probe. In azoaldolase the location of diazo thioethers along the peptide chain is known.[3] The involved residues, Cys 237 and Cys 287, have different chemical properties: they are both considered "exposed" to the solvent, but the first is definitely more reactive than the second.[23] The p-carboxylate azo chromophore formed on Cys 237, having isomerization rates similar to that of the model in aqueous solution, confirms the complete exposition of the amino acid residue to the solvent; the slower isomerization rates found for the diazo thioether formed on Cys 287 indicate that the residue is located in a less polar environment.[5]

Double exponential kinetics were not identified in the p-nitrobenzeneazoaldolase. In this case, though the protein matrix markedly affects the rates of the thermal isomerization, there is no selective effect of the protein microenvironment on the kinetic properties of the azo chromophores. This could be related to the nature of the isomerization mechanism which is operating on the protein. However, the different behavior of the p-carboxylate benzenediazo thioethers could be also related to the presence of a free charge which can introduce interactions with specific groups of the protein. In addition the p-carboxylate diazo thioether has been observed to play a specific role in inducing modifications of the electrophoretic mobility of azoaldolase under light control,[2b] and in regulating the catalytic activity of the modified enzyme within a light-dark cycle.[24]

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