Table III: Labeling of Purified F_1 Subunits with Radioactive *N*-Ethylmaleimide.[a]

Subunit	mol of MalNEt Bound/mol of Protein
$F_1 - 1$	4.08, 3.96
$F_1 - 2$	0.17, 0.20
$F_1 - 3$	0.91; 0.88; 0.81, 0.78
$F_1 - 4$	0.04; 0.1
$F_1 - 5$	0.81; 0.78

[a] Lyophilized subunits were dissolved in 1% SDS-40 mM Tris-SO_4-1 mM EDTA-50 mM DTT (pH 8.0) at 5-8 mg/ml. Further treatment was as described in Table I, superscript *a*. Molecular weight values for subunits used in calculation were those suggested as "best values" currently available (Senior 1973a, p 255).

cule. Combining the data presented in Tables I, II, and III, the results obtained using this technique of labeling with radioactive MalNEt indicate that there are two molecules of subunit 1, two molecules of subunit 3, and two molecules of subunit 5 per molecule of F_1. Thus this technique gives results which are in conflict with previous suggestions. Present work in this laboratory is aimed at development of other techniques which will enable the stoichiometry of subunits 2 and 4 to be investigated, and which might allow application of the general principle of this method to examination of other membrane-bound enzyme complexes, such as electron-transfer complexes.

Acknowledgment

I thank Christine Broadhurst for good technical help.

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Modification of the Cysteine Residue of Streptococcal Dihydrofolate Reductase†

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ABSTRACT: Modification of the single cysteine residue of streptococcal dihydrofolate reductase with 5,5′-dithiobis(2-nitrobenzoic acid) results in virtually complete inactivation of the enzyme. Reduction of the enzyme-*S*-nitrobenzoate mixed disulfide with dithiothreitol indicates that 1.0 cysteine has been modified and approximately 70% of the original enzyme activity restored. Circular dichroic and fluorescence studies suggest that a localized conformational change involving aromatic residues, possibly tryptophan, has occurred following modification. In addition, nearly stoichiometric amounts of *p*-hydroxymercuribenzoate lead to complete inactivation of the reductase. It is suggested that the *S*-nitrobenzoate moiety bound to the cysteine residue of the enzyme may perturb one or more aromatic side chains and lead to a distortion of the hydrophobic substrate binding site.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduced triphosphopyridine nucleotide-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, the coenzyme involved in many one-carbon transfer reactions. This enzyme appears to be the primary target for the action of 4-amino analogs of folate, such as aminopterin (4-amino-4-deoxyfolate) and amethopterin (4-amino-10-methyl-4-deoxyfolate). The latter compound has been widely used in the chemotherapeutic treatment of certain leukemias, lymphomas, and other clinical disorders (Huennekens, 1968; Blakley, 1969).

Although a considerable amount of work has been done on the design and synthesis of folate antagonists (reviewed by Blakley, 1969), little is known about the mechanism of

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action of the enzyme in terms of functional groups involved in interactions with substrates and inhibitors. This type of information is often obtained by chemical modification of certain amino acid side chains of the particular enzyme under study and observing the effect of the modification upon the enzyme-catalyzed reaction and on the structure of the protein. The ultimate success of this procedure depends upon the specificity and completeness with which a single functional group can be modified.

In previous investigations tryptophan residues have been implicated in substrate and inhibitor binding in dihydrofolate reductases from chicken liver (Freisheim and Huennekens, 1969) and from amethopterin-resistant strains of *S. faecium* (Warwick *et al.*, 1972), *Escherichia coli* (Greenfield *et al.*, 1973) and *Lactobacillus casei* (Liu and Dunlap, 1974). In a preliminary study Greenfield *et al.* (1973) suggest the functional involvement of histidine residues at the active site of the reductase from amethopterin-resistant *E. coli* based on chemical modification studies with diethyl pyrocarbonate. In another report Gleisner and Blakley (1973) suggest the catalytic involvement of one of two methionine residues modified following iodoacetate treatment of the reductase from an amethopterin-resistant mutant of *S. faecium*. The latter strain appears to be different from that employed in this study as discussed previously (D'Souza *et al.*, 1972).

The present study was undertaken in order to examine the possible function of the single cysteine residue of streptococcal dihydrofolate reductase in our continuing investigation of amino acid residues involved in substrate and inhibitor binding.

Experimental Section

Materials. Dihydrofolate reductase was isolated and purified from an amethopterin-resistant strain of *Streptococcus faecium* according to the procedure of D'Souza *et al.* (1972). TPNH and dithiothreitol were purchased from P-L Biochemicals. Nbs_2[1] was purchased from Aldrich Chemical Co. HgBzOH was obtained from Sigma Chemical Co. Dihydrofolic acid was prepared by the reduction of folic acid (Calbiochem) with sodium dithionite according to the method of Futterman (1957), as modified by Blakley (1960). Dihydrofolic acid was washed repeatedly with 5×10^{-3} N HCl, lyophilized, and stored in evacuated, sealed tubes under argon. All other chemicals were of reagent or analytical grade.

Methods. Dihydrofolate reductase activity was measured at 25° by monitoring the decrease in absorbance at 340 nm using a Beckman Model DU spectrophotometer equipped with a Gilford Model 208 auxillary offset control unit, a Gilford Model 210 automatic cuvet positioner, and a Sargent Model SRLG recorder. The standard 1.0-ml assay contained 50 mM KPO_4 (pH 6.5), 50 μM TPNH, and 33 μM dihydrofolate.

Protein concentrations were determined either by measuring the absorbance at 215 and 225 nm, according to the method of Waddell (1956), or by titration of the enzyme with amethopterin, a stoichiometric inhibitor of the enzyme (Werkheiser, 1961) as outlined by D'Souza *et al.* (1972).

Circular dichroic spectra were measured at 25° in a Cary Model 61 spectropolarimeter. Ultraviolet absorption spectra were measured on a Cary Model 15 spectrophotometer.

[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); HgBzOH, *p*-hydroxymercuribenzoate.

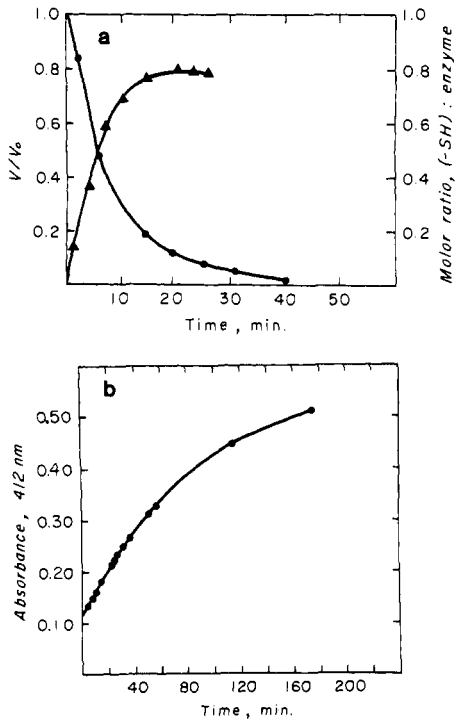

FIGURE 1: (a) Inactivation of dihydrofolate reductase by Nbs_2 at 39°. The molar ratio of Nbs_2/enzyme is 25:1. Enzyme activity, V/V_0, is expressed as a fraction of an untreated control (●- - -●). The molar ratio of -SH groups reacting per mole of enzyme (▲- - -▲) was calculated from the concentration of released anion (see Methods). (b) Hydrolysis of Nbs_2 (5×10^{-4} M) at 39° in 0.1 M KPO_4 (pH 8.0). The concentration of released anion *vs.* time was measured at 412 nm.

Fluorescence spectra were measured at 25° in a Fluorispec fluorescence spectrophotometer equipped with a Bausch & Lomb Omnigraphic 2000 recorder.

Modification of the cysteine residue of dihydrofolate reductase by Nbs_2 was monitored at 412 nm (Ellman, 1959). The reaction was initiated by the addition of Nbs_2 to 1.0-ml cuvets containing enzyme (2×10^{-5} M) in 0.1 M KPO_4 (pH 8.0) and to a buffer blank. The temperature was maintained at 39° and the molar ratio of Nbs_2/enzyme was 25:1. Aliquots of 5–10 μl each were removed from the cuvet containing enzyme at various time intervals and assayed for catalytic activity. The number of enzyme sulfhydryl groups modified was quantitated employing a molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} at 412 nm for the released anion (Ellman, 1959). In the calculations the absorbance at 412 nm due to anion release in the Nbs_2–enzyme reaction was corrected for that which occurred in the Nbs_2–buffer control at the same time interval. The fractional activity remaining is expressed as V/V_0, where V is the enzyme activity of the Nbs_2-modified reductase and V_0 is the activity of an unreacted control.

Results

Effect of Nbs_2 on Enzyme Activity. The results of the reaction of streptococcal dihydrofolate reductase with Nbs_2 are shown in Figure 1a. As indicated in the figure the number of sulfhydryl groups modified appears to level off at 0.8 when the enzyme is inactivated to the extent of *ca.* 92%. Examination of the buffer blank containing only Nbs_2 (Figure 1b) shows, however, that the release of the colored anion is not strictly linear and, thus, the number of sulfhydryl groups modified based solely on anion release must be considered as only an approximation. Therefore, the degree of

Table I: Substrate Protection against Inactivation of Dihydrofolate Reductase by Nbs_2.[a]

Molar Ratio TPNH/Enzyme	V/V_0	Molar Ratio Dihydrofolate/Enzyme	V/V_0
0	0.28	0	0.23
20	0.99	15	0.48
50	1.03	50	0.90

[a] The enzyme (1.0×10^{-6} M in 0.10 M Tris-HCl (pH 8.0)) was incubated at 37° with either TPNH or dihydrofolate in a total volume of 1.0 ml. Each reaction solution contained 6.2×10^{-5} M Nbs_2. The results shown were obtained following incubation for 2 hr.

-SH modification following Nbs_2 modification was examined in the inactivated enzyme. The reductase (1.50×10^{-5} M) was inactivated (*ca.* 95%) under similar conditions to those described in Figure 1a, dialyzed for 48 hr *vs.* two 4 l. changes of 0.05 M KPO_4 (pH 7.5) to remove excess Nbs_2 and incubated with 0.03 M dithiothreitol. The absorbance at 412 nm was measured after 30 min at 25° to measure the amount of anion released from the protein following disulfide reduction. The concentration of the librated anion was calculated to be 1.48×10^{-5} M (see Methods). Thus, inactivation of streptococcal dihydrofolate reductase with Nbs_2 correlates with the modification of 1.0 cysteine residue in the protein.

Substrate Protection against Nbs_2 Inactivation. The results of incubation of the enzyme with either of the substrates, TPNH or dihydrofolate, prior to treatment with Nbs_2 are indicated in Table I. Both TPNH and dihydrofolate appear to substantially protect the enzyme against modification by Nbs_2. At molar ratios of TPNH or dihydrofolate to enzyme of 15–20:1 TPNH affords complete protection, whereas the enzyme is inhibited *ca.* 50% in the presence of dihydrofolate. There is no apparent effect of Nbs_2 on either substrate as determined by monitoring the change in absorbance at 340 nm upon incubation of either substrate in the presence or absence of Nbs_2 for 2 hr at 37°. No difference in absorbance at 340 nm was observed in the samples containing Nbs_2 from those containing substrate alone.

Uv Absorption Spectrum of the Nbs_2-Modified Enzyme. The uv absorption spectrum of Nbs_2-modified dihydrofolate reductase (Figure 2) shows a maximum at 278 nm while that of the unmodified enzyme shows a maximum at 277 nm. Both modified and unmodified proteins show a shoulder at *ca.* 290 nm. In addition, the Nbs_2-modified reductase exhibits a second broad peak with a maximum in the 310–320 nm region which is due to the Nbs_2–enzyme.

Circular Dichroic Spectrum of the Nbs_2-Modified Enzyme. Previous circular dichroic studies from this laboratory concerning the binding of various substrates and inhibitors to streptococcal dihydrofolate reductase have shown that the large aromatic side-chain Cotton effect exhibited by the enzyme is sensitive to changes at or near the active site (D'Souza and Freisheim, 1972). Thus, this intrinsic Cotton effect is particularly useful for examining possible conformational differences between the modified and native enzymes.

The circular dichroic absorption spectra of the native and Nbs_2-modified enzymes in the 240–350-nm region are shown in Figure 3. As indicated in the figure, the side-chain

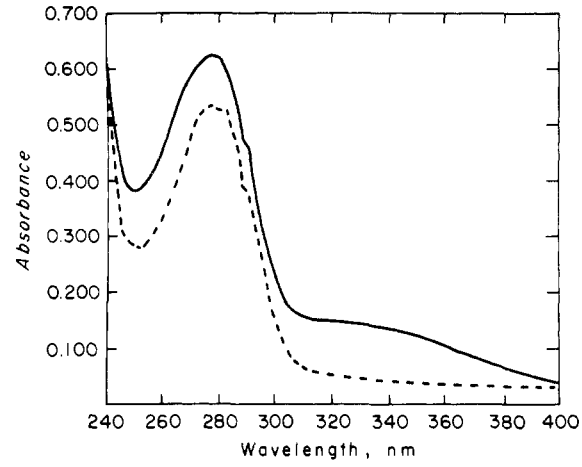

FIGURE 2: Comparison of the ultraviolet absorption spectra of Nbs_2-modified (—) and native (- - -) dihydrofolate reductases. Enzyme (1.5×10^{-5} M) was inactivated as described in Figure 1 and dialyzed *vs.* two changes of 4 l. each of 0.05 M KPO_4 (pH 7.5) for 48 hr prior to analysis.

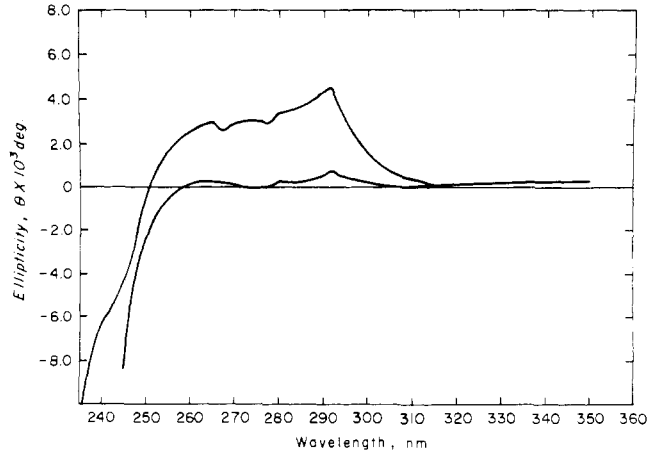

FIGURE 3: Effect of Nbs_2 modification on the circular dichroic spectrum of dihydrofolate reductase in the 240- to 350-nm region. The native enzyme, 2.3×10^{-5} M in 0.05 M KPO_4 (pH 7.5) (upper curve), is treated with Nbs_2 as described in Figure 1 and dialyzed *vs.* two changes of 0.05 M KPO_4 (pH 7.5) for 48 hr.

Cotton effect in the 260–310-nm region exhibited by the native enzyme is virtually abolished following Nbs_2 inactivation. Comparison of the circular dichroic spectrum of the native enzyme with that of the inactivated enzyme in the far-uv region suggests that no gross alterations in the polypeptide backbone have occurred following Nbs_2 modification (Figure 4). As shown in the figure, the modified enzyme displays a more negative ellipticity at 225 nm than does the native enzyme. The loss of the small positive Cotton effect centered at *ca.* 235 nm may, in fact, account for the differences observed at 225 nm.

Fluorescence Spectrum of the Nbs_2-Modified Enzyme. The fluorescence spectra of tryptophan containing proteins are sensitive to the tertiary structure of the protein and to the microenvironment of tryptophan. Previous studies have shown that the fluorescence spectrum of dihydrofolate reductase is sensitive to substrate and inhibitor binding to the enzyme and thus reflects events occurring at the active site of the enzyme (Perkins and Bertino, 1966; Huennekens *et al.*, 1971; Gundersen *et al.*, 1972; Erickson and Mathews, 1973; Williams *et al.*, 1973a,b). The fluorescence spectrum of the native reductase was compared to the spectrum of the

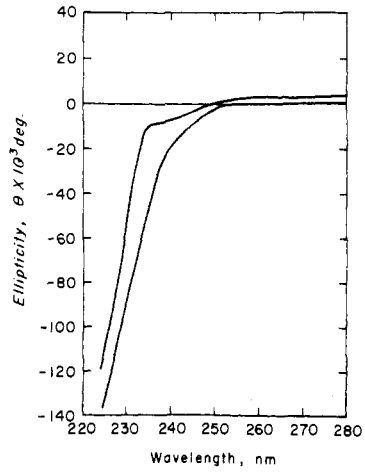

FIGURE 4: Far-uv circular dichroic spectra of native (upper curve) and Nbs_2-modified dihydrofolate reductase. Other conditions are given in the legend to Figure 3.

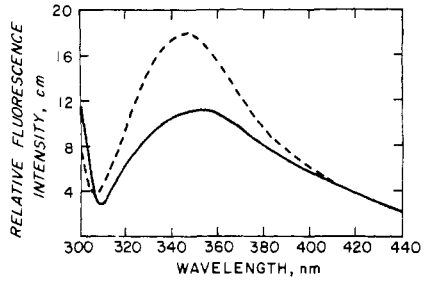

FIGURE 5: Fluorescence emission spectra of native (- - -) and Nbs_2-modified (—) dihydrofolate reductase. The enzyme concentration is 4.5×10^{-7} M in 0.05 M KPO_4 (pH 7.5). The excitation wavelength is 290 nm. Other conditions are given in the legend to Figure 3.

Nbs_2-modified enzyme (Figure 5). While the native enzyme has an emission maximum at 345 nm when excited at 290 nm, the Nbs_2-modified enzyme has an emission maximum at 350 nm. In addition, the relative fluorescence intensity of the Nbs_2-modified enzyme has been reduced by *ca.* 34% when compared with that of the native enzyme. The fluorescence data are in agreement with the circular dichroic studies which suggest that the Nbs_2-modified enzyme has undergone a definite conformational change as signalled by an alteration in the environment of one or more aromatic amino acid residues. However, the circular dichroic data also indicate that a general unfolding of the polypeptide backbone has not occurred since the ellipticity at 225 nm does not increase following Nbs_2 modification.

Reactivation of the Nbs_2-Modified Reductase. Attempts to reactivate the Nbs_2-modified enzyme are also consistent with the view that while localized conformational changes have occurred a general denaturation has not. Approximately 2 mg of dithiothreitol was added to 0.35 ml of Nbs_2-modified dihydrofolate reductase (1×10^{-6} M in 0.1 M KPO_4 buffer (pH 8.0)) which had *ca.* 10% enzyme activity remaining. Aliquots of 5–10 μl were subsequently removed and assayed at various times. Maximum recovery of enzymatic activity (70%) was observed after approximately 25 min. The dithiol had no effect on unmodified reductase under these conditions.

Inactivation of Dihydrofolate Reductase by HgBzOH. In addition to examining Nbs_2 modification of dihydrofolate reductase in some detail, the effect of the mercurial HgBzOH on enzymatic activity was also investigated.

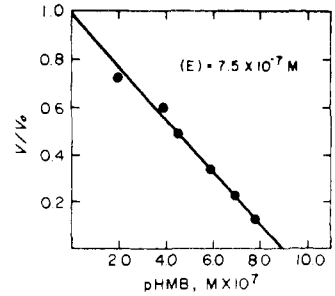

FIGURE 6: The effect of HgBzOH on dihydrofolate reductase activity. Enzyme inactivation at 39° was followed by the addition of 10-μl aliquots of the reagent (9.8×10^{-5} M) to a 1-ml solution of the reductase (7.5×10^{-7} M in 0.01 M KPO_4 (pH 7.0)). The reaction was allowed to proceed for 20 min after each addition at which time aliquots were removed and assayed.

As shown in Figure 6 there is a linear inactivation of dihydrofolate reductase in the presence of low concentrations of HgBzOH. Complete inactivation of the enzyme occurs at virtually stoichiometric concentrations of reductase and mercurial.

Discussion

As reviewed elsewhere (Huennekens, 1968; Blakley, 1969) agents that react with sulfhydryl groups increase the catalytic activity of most, but not all, animal dihydrofolate reductases from *ca.* two- to fivefold. An interesting corollary to the activation process is that the double pH optima observed for each of the native enzymes is shifted to a single optimum. Organomercurial compounds produce such increases in catalytic activity in reductases from Ehrlich ascites cells (Perkins and Bertino, 1965) and from chicken liver (Kaufman, 1964). Iodine (Kaufman, 1966) and *N*-bromosuccinimide (Freisheim and Huennekens, 1969) have been shown to result in similar increased chicken liver reductase activity. The evidence for the involvement of sulfhydryl groups in these cases was based largely on the reversal of the higher enzyme activities to normal levels following treatment with thiols such as mercaptoethanol or dithiothreitol. Activation of dihydrofolate reductase from L1210 lymphoma cells can also be effected by treatment with Nbs_2 (Reyes and Huennekens, 1967) and reversed by a thiol.

By contrast several bacterial dihydrofolate reductases exhibit a single pH optimum, are not activated by these sulfhydryl reagents, and, in fact, may be inhibited by such compounds (reviewed by Huennekens *et al.*, 1971). The molecular relationships between catalytic activation involving a sulfhydryl group, or lack of it, and a double or single pH optimum remains to be elucidated. Thus, based on animal *vs.* bacterial enzyme differences, one must be cautious in assuming that the properties of any given reductase can be predicted from the properties of another.

Since the reversible inactivation of dihydrofolate reductase by Nbs_2 is coincident with the modification of one cysteine residue, and since both the substrates, TPNH and dihydrofolate, protect the enzyme from this inactivation, it would appear likely that this cysteine is at or near the active site of the enzyme. Alternatively, it could be argued that binding of the substrates causes a conformational change distant to the active site which buries the sulfhydryl and renders it unavailable for reaction.

The spectral investigations, particularly the circular dichroic data, suggest that while no gross denaturation of the

enzyme occurs, the active site may have undergone a localized conformation change as indicated by the virtual abolition of the aromatic side-chain Cotton effect. The origin of the positive Cotton effect centered at *ca.* 235 nm of streptococcal dihydrofolate reductase is unknown. The aromatic amino acid residues phenylalanine, tyrosine, and tryptophan as well as cystine may exhibit optically active absorption bands in the 240–300-nm region (reviewed by Adler *et al.*, 1973). These chromophores have absorption bands in the 185–240-nm region as well and may be optically active in a protein, particularly if the side chain exists in an asymmetric environment. Cystine can be eliminated since none occurs in the enzyme, but any or all of the aromatic residues may contribute to the 235-nm ellipticity band.

The reduction in fluorescence intensity of the Nbs_2 modified enzyme may be due to quenching of fluorescence of tryptophan in the enzyme by the bound *S*-nitrobenzoate-moiety substantiating the premise that one or more Trp residues previously implicated in the active center of the enzyme may be proximal to the reacting -SH. The shift in the fluorescence emission maximum in the modified enzyme may be due to the loss of fluorescence of one or more Trp residues due to quenching leading to the isolation of other Trp residues in the enzyme with higher fluorescence maxima.

Alternatively, the fluorescence data may be indicative of a conformational change occurring in the enzyme upon Nbs_2 modification leading to a change in the environment of one or more Trp residues in the enzyme and the observed differences in the fluorescence spectra of the native and modified enzymes. This interpretation is in agreement with the CD studies which suggest that the Nbs_2-modified enzyme has undergone a definite conformational change signalled by an alteration in the environment of one or more aromatic amino acid residues.

Our earlier work on *N*-bromosuccinimide-modified dihydrofolate reductase showed a pronounced decrease in the magnitude of the 290-nm CD band which coincided with the modification of two active site tryptophans (Warwick *et al.*, 1972). We proposed that the two indolyl moieties of the tryptophans may exist in a stacked arrangement through π-π interactions creating a hydrophobic binding pocket for the aromatic portions of the two substrates. Such an argument is enhanced by the observations that reduced or oxidized folates, including amethopterin, intensify the aromatic side-chain Cotton effect displayed by the enzyme (D'Souza and Freisheim, 1972). For example, the maximum total change in molar ellipticity at 290 nm for the dihydrofolate–enzyme complex is $+828{,}000°$ cm^2 $dmol^{-1}$.

Based on these findings the *S*-nitrobenzoate moiety bound to the cysteine residue following Nbs_2 modification may perturb one or two of the tryptophan residues previously implicated in the active site of the enzyme. Such an interaction could lead to a distortion of the active site as indicated by the loss of the aromatic side-chain Cotton effect and the quenching of the protein fluorescence following Nbs_2 modification.

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