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Modification of Myosin Subfragment 1 Tryptophans by Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium Bromide†

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ABSTRACT: Modification of tryptophanyl residues (Trps) of myosin subfragment 1 (S-1) was performed with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (DHNBS). Under controlled conditions, pH 6 at 0 °C and 10-min reaction with 10–100-fold molar excess, K^+(EDTA) activity was reduced down to less than half, whereas Ca^{2+}-ATPase activity increased and acto-S-1-ATPase was not affected. The number of modified Trps (up to 2.5) agreed well with the number of 2-hydroxy-5-nitrobenzyl moieties incorporated in S-1. The thiol groups of S-1 were not affected up to 50-fold molar excess of DHNBS, thus indicating that the modification was selective for Trps. The modification of as few as one Trp caused a blue shift of the emission spectrum, accompanied by a reduction in the fluorescence quantum yield. The accessibility of Trps to the fluorescence quencher acrylamide is drastically reduced upon modification, indicating that DHNBS-reactive Trps are more "exposed" than the DHNBS-refractive ones. DHNBS modification did not seem to affect the ATP-induced tryptophan fluorescence enhancement of S-1. The effect of DHNBS modification on the intrinsic fluorescence of S-1 indicates that the modified Trps are located in a polar environment and that they may be identical with the long-lifetime Trps of Torgerson [Torgerson, P. (1984) *Biochemistry 23*, 3002–3007]. The most reactive Trp is located in the N-terminal 27-kDa fragment of the S-1 heavy chain. It might also be inferred from the above data that the nonexposed and ATP-perturbed Trp(s) is (are) located in the 50-kDa fragment.

The energy required for the contraction process is derived from hydrolysis of ATP, which occurs at a remote ATP binding site of myosin. This energy is transferred to the actin binding site, most probably in the form of a structure distortion through the S-1[1] segment, which serves as a transducer in the process (Botts et al., 1984). Such a model requires the existence of an intersite communication system. To trace this system, one should study environment-sensitive functional groups, which have the capability to "report" on conformational changes taking place in their vicinity. Trps, having environment-sensitive absorbance and fluorescence spectra, can serve as intrinsic reporter groups and have been widely used in the study of myosin structure and function. Morita

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[1] Abbreviations: ϵ_M and $\epsilon_{1\%}$, absorption coefficients, molar and by percent; DHNBS, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTE, dithioerythritol; DTT, dithiothreitol; GdmCl, guanidinium chloride; HMM, heavy meromyosin; HNB, 2-hydroxy-5-nitrobenzyl; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; kDa, kilodaltons; P_i, inorganic phosphate; S-1, chymotryptic subfragment 1; S-2, myosin subfragment 2; $NaDodSO_4$, sodium dodecyl sulfate; TES, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; Trp, tryptophanyl residue; PAGE, polyacrylamide gel electrophoresis; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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(1967) was the first to observe a UV difference spectrum upon addition of nucleotide to myosin due to the perturbation of the absorbance spectrum of one or two Trps. Werber et al. (1972) found an increase in the fluorescence emission spectrum of Trps upon addition of nucleotides and examined the relationship between the structural features of nucleotides and the amplitude of the emission increment. They also observed that those Trps, whose emission spectra were perturbed by addition of nucleotides, were inaccessible to iodide quenching. The tryptophan fluorescence increment observed upon addition of nucleotides has been amply exploited in the study of the detailed mechanism of the myosin-catalyzed ATP hydrolysis (Trentham et al., 1976; Taylor, 1979). Recently, it was suggested by Okamoto and Yount (1985) that one Trp, Trp-130, in the sequence of S-1 heavy chain (Tong & Elzinga, 1983), might be directly involved in the binding of the adenylate moiety of ATP (Okamoto & Yount, 1985). However, in spite of these advances, virtually nothing is known about the location of those Trp(s), whose emission is specifically altered during binding and hydrolysis of ATP.

In light of the important information obtained from the study of Trps concerning myosin function, it is obvious that these residues should be individually characterized, especially in connection with the conformational changes taking place during the ATPase cycle (Chock et al., 1979). One approach to this problem was to resolve the intrinsic emission of the five Trps of S-1 into three classes on the basis of their decay-associated spectra (Torgerson, 1984). In that work it was found that the fluorescence of the intermediate-lifetime component was enhanced upon addition of ATP and that only the long-lifetime component was accessible to acrylamide quenching. Analysis of the three Trps of the 50-kDa tryptic fragment of S-1 showed that the acrylamide-accesible long-lifetime component is missing from this fragment (Muhlrad et al., 1986). It seemed of interest to combine the spectroscopic characterization with selective chemical modification, and therefore, we have sought in this work to modify S-1 Trps with a water-soluble reagent, DHNBS (Horton & Tucker, 1970). We have found that even at a high molar excess of DHNBS, only two to three out of the five Trps of S-1 heavy chain are accessible to the reagent. The modification caused a change in the intensity and shape of the intrinsic fluorescence spectrum and an alteration of the ATPase activity. It is also shown that the first Trp, which is modified, is located in the N-terminal 27-kDa tryptic fragment of S-1.

Materials and Methods

Chemicals. DHNBS (bromide salt), DTE, DTT, DTNB, IAEDANS, ATP, and ADP were from Sigma. TPCK-trypsin, soybean trypsin inhibitor and α-chymotrypsin were purchased from Millipore. These and all other chemicals were of reagent grade.

Preparation of Proteins. Myosin and actin were prepared from the back and leg muscles of rabbits according to the method of Tonomura et al. (1966) and Spudich and Watt (1971), respectively. S-1 was prepared by the digestion of myosin filaments with α-chymotrypsin by the method of Weeds and Taylor (1975) except that soybean trypsin inhibitor was also added at $^1/_{10}$ of the chymotrypsin concentration to inhibit traces of trypsin.

Modification of S-1 with IAEDANS. Labeling of S-1 with IAEDANS was essentially performed as described by Takashi et al. (1976). IAEDANS (10-fold molar excess) was added to S-1 in 150 mM KCl and 10 mM TES, pH 7.0, and after 1-h incubation at 0 °C, the reaction was quenched by a 10-fold molar excess of DTE over IAEDANS. The amount of IAEDANS bound to S-1 was estimated from the absorbance at 338 nm (ϵ_M = 6300 M^{-1} cm^{-1}) after the unreacted IAEDANS was dialyzed out.

Modification of S-1 by DHNBS. S-1 was modified at 0 °C (on ice) at pH 6.0 in histidine hydrochloride buffer (20 mM) by the addition of aliquots of an aqueous solution of 25 mM DHNBS. The concentration of DHNBS was determined in the presence of 0.1 N NaOH at 415 nm, ϵ_M = 18 400 M^{-1} cm^{-1} (Barman & Koshland, 1967). The concentration of S-1 varied between 3.0 and 5.5 mg/mL, and the molar excess of DHNBS ranged from 10- to 150-fold. After incubation for 10 min, the reaction was quenched by incubation in 5 mM DTE or DTT (final concentration) for 30 min at 0 °C. The samples were then immediately exhaustively dialyzed 4 times (against two changes of 20 mM Tris-HCl, 150 mM KCl, and 1 mM EDTA, pH 7.8, and two changes of 20 mM Tris-HCl, 30 mM NaCl, and 1 mM EDTA, pH 7.8). The dialyzed samples were applied to a Sephadex G-50 column (0.6 × 45 cm) equilibrated with the last dialyzing buffer.[2] The protein content and the 415-nm absorption (at pH 12) of 2.2-mL aliquots of the eluent fractions were measured. The protein peaks were used for further enzymatic and fluorescence studies.

Preparation of the Tryptic Fragments of S-1 Heavy Chain. The 20-kDa and 50-kDa fragments were prepared from the tryptic digest of S-1 as described in our earlier work (Muhlrad & Morales, 1984). The 27-kDa fragment was also prepared from the tryptic digest of S-1. The heavy chain fragments were dissociated by 6 M GdmCl, and then the 27-kDa fragment was precipitated together with a part of the 50-kDa fragment by addition of 3 volumes of ethanol. The precipitate was collected by centrifugation and dissolved in 1% $NaDodSO_4$, 4 mM mercaptoethanol, and 10 mM Tris–acetate, pH 7.8. Finally, the pure 27-kDa fragment was obtained by filtering the dissolved precipitate through a Sephadex G-100 column equilibrated with the buffer used for the solubilization except the concentration of $NaDodSO_4$ was 0.1%.

ATPase Assays. ATPase activities [μmol of P_i (mg of S-1)$^{-1}$ min^{-1}] were calculated from the P_i content (Fiske & Subarrow, 1925). The reaction was carried out at 25 °C in 1-mL timed aliquots containing 10–20 μg of S-1 and 2 mM ATP in 600 mM KCl, 50 mM Tris-HCl, pH 8.0, and either 6 mM EDTA [for K^+(EDTA)-activated ATPase] or 6 mM $CaCl_2$ (for Ca^{2+}-activated ATPase). Actin-activated ATPase activity of S-1 was measured at 25 °C in 1-mL timed aliquots containing 11.5 μg of S-1, 21 μg of F-actin, 1 mM ATP, 2 mM $MgCl_2$, and 20 mM imidazole, pH 7.0. Incubation times were chosen so that less than 15% of the ATP was hydrolyzed.

Fluorescence measurements were performed at 25 ± 0.2 °C on a Perkin-Elmer spectrofluorometer, Model MPF 44 or SLM Model 8000, interfaced with an IBM-XT computer in the ratio recording mode. The excitation wavelength selected was 295 nm so as to ensure that only Trps were excited (Werber et al., 1972). The concentration of S-1 was between 20 and 100 μg/mL (for native S-1, the absorbance at 295 nm was below 0.02 OD unit). The introduction of several HNB

[2] In the first stage of this work the excess of reagent and the HNB residues bound noncovalently to S-1 were removed by four consecutive dialyses to which we subjected the modified protein after quenching the reaction with DTT. However, in some cases we observed that the ratios of HNB:Trp were higher than the maximal number of Trps in chymotryptic S-1, which is five (Tong & Elzinga, 1983; Torgerson, 1984). As the molar excess of DHNBS increased, larger excesses of noncovalently bound reagent were found to remain associated with the labeled protein, resulting in erroneous values of the HNB:Trp ratio (Werber & Muhlrad, 1986). We therefore separated the labeled protein from the excess reagent (which could not be removed by dialysis) by gel filtration.

groups in the protein affects its absorbance (Naik & Horton, 1973). Therefore, the fluorescence efficiency of the various modified S-1s was not obtained by dividing the fluorescence intensities by their absorbances but rather by their protein concentrations, and this ratio was termed "specific fluorescence intensity". In measuring the effect of nucleotides on the fluorescence, the results were expressed as the ratio [$\Delta I/I$ (in percent)] of the increase in fluorescence intensity, ΔI, at the peak of the spectrum induced by the effector to the absolute intensity measured in its absence, I (Werber et al., 1972). The fluoresence intensity was corrected for dilution effects caused by the addition of aliquots of stock solutions of metal ions and nucleotides.

Extent of Modification. The concentration of incorporated HNB groups was determined at 415 nm as described above. An alternative method to estimate the extent of modification was also employed; here we determined by intrinsic fluorescence the residual Trp content of the modified S-1 samples that had been denatured by 6 M GdmCl (Naik & Horton, 1973) in 8.5 mM EDTA, 85 mM KCl, and 42 mM Tris-HCl, pH 7.8. The fluorescence was measured at 25 °C; excitation was at 295 nm, and emission was read at 350 nm. The method was calibrated by using as standard *N*-acetyltryptophanamide, whose concentration had been determined from its absorbance at 280 nm, ϵ_M = 5600 M^{-1} (Fasman, 1976). At the protein concentration used (<1 μM) inner filter effects of Trp-bound HNB were found to be negligible. The number of modified residues per S-1 was calculated (directly in the first method and by substraction from the control value in the second one) on the basis of an M_r of 115 000 for S-1 (Margossian et al., 1981).

Thiol Content. Thiol groups were determined via a modified Ellman (1959) procedure: Titration with DTNB was performed at pH 7.0, following denaturation of the protein in 6 M GdmCl at pH 8.0. Appropriate corrections accounting for the HNB absorbance at 412 nm were made for each sample.

Protein concentration was estimated by the Bradford (1976) method or by a modified Bradford technique (Macart & Gerbault, 1982), using as a standard S-1, whose concentration had previously been determined from its absorbance at 280 nm, $\epsilon_{1\%}$ = 7.5 (Wagner & Weeds, 1977). When the samples were in a 0.1% $NaDodSO_4$ and 4 mM β-mercaptoethanol containing solution, then the method of Lowry et al. (1951) was used for protein determination. In this case, 8 mM *N*-ethylmaleimide was added prior to the measurement, in order to quench the free thiol groups of mercaptoethanol.

Acrylamide Quenching. To a cuvette containing 2.5 mL of a sample of control or DHNBS-modified S-1, 20–100 μg/mL S-1 in 20 mM Tris-HCl, 30 mM NaCl, and 1 mM EDTA, pH 7.8, were added consecutive aliquots of a fresh acrylamide solution, giving rise to final concentrations between 0 and 110 mM. The fluorescence intensity was measured at each acrylamide concentration under the same conditions as above.

Results

The time course of the modification of S-1 with DHNBS at pH 6.0 at two molar excesses of modifying reagent (25 and 150) was monitored by measuring the incorporation of HNB residues in the protein. The results of this preliminary experiment (not shown) indicated that after 10 min at pH 6.0 the reaction was virtually completed, and therefore, these were the conditions used in all further experiments.

The dependence of both the number of HNB groups incorporated in S-1 and the number of Trps modified on the molar excess of DHNBS is shown in Figure 1. Good agreement exists between the two methods of monitoring the modification, and the maximal number of residues modified is around 2.5. Under the conditions of the modification (pH 6 at 0 °C) the cysteine residues of S-1 are not significantly affected up to a molar excess of 100-fold of DHNBS (Table I).

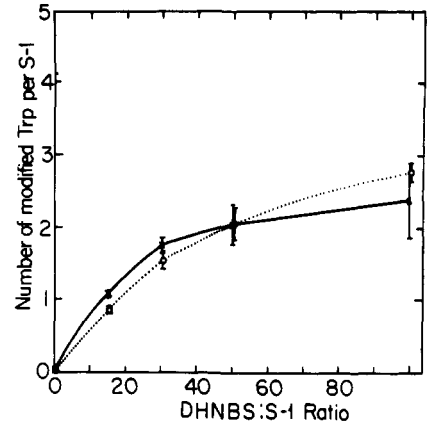

FIGURE 1: Number of HNB groups introduced and Trp(s) modified in S-1 as a function of the molar ratio DHNBS:S-1. For conditions of modification and measurements, see Materials and Methods. (○) Number of HNB groups incorporated per S-1; (×) number of tryptophans modified. (Vertical bars) Standard error. (Abscissa) DHNBS to S-1 molar ratio during modification (mean of six experiments).

Table I: Effect of DHNBS Modification of S-1 on the Number of Thiol Groups

molar excess	number of HNB groups incorporated per S-1[a]	number of thiol groups per S-1
0	0	9.4
15	0.9–1.0	9.4
30	1.5	9.2
50	1.9	9.3
100	2.4	8.4
150	2.5	8.2

[a] Average from the determination of the number of Trps whose fluorescence is quenched and of the number of HNB residues bound, according to their absorbance in alkali.

The changes in the ATPase activities [Ca^{2+}, K^+(EDTA), and actin mediated] as a function of the molar excess of DHNBS are shown in Figure 2. Whereas K^+(EDTA)-ATPase is more than half-reduced at the highest excess of DHNBS tested (100-fold), Ca^{2+}-ATPase is increased and acto–S-1-ATPase is not affected. Since it is well-known that the labeling of the reactive SH_1 thiol of S-1 (Sekine & Yamaguchi, 1963) causes also an increase in Ca^{2+}-activated and a decrease in K^+(EDTA)-activated ATPase activities, we studied the effect of DHNBS modification on the ATPase activities of S-1, whose SH_1 thiol was premodified with IAEDANS (Takashi et al., 1976). As it is shown in Figure 3, the DHNBS modification of the Trps of the SH_1-blocked S-1 leads to a decrease both in Ca^{2+}- and in K^+ (EDTA)-activated ATPase activities.

Modification of S-1 by DHNBS, even at low molar excess, when only one Trp is modified [resulting in 80% and 110%, respectively, of the K^+(EDTA)- and Ca^{2+}-ATPase activities of control S-1], causes a change in the shape of the intrinsic fluorescence emission spectrum with a blue shift of the maximum (Figure 4). This changes, as expressed by the ratio of the fluorescence intensities at two wavelengths (340 nm and the maximum), becomes gradually more pronounced with the extent of modification (Figure 5). Concomitant with the change in the spectrum shape, there occurs a decrease in the

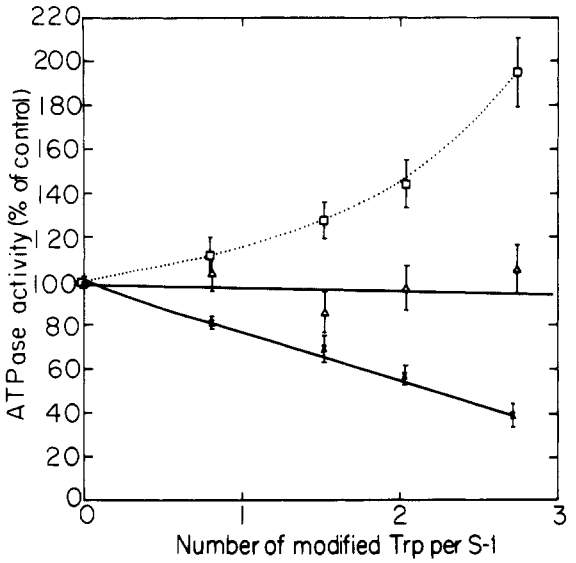

FIGURE 2: Effect of DHNBS modification on the ATPase activity of S-1: (×) K^+(EDTA)-activated, (□) Ca^{2+}-activated, and (△) actin-activated ATPase activities. (Vertical bars) Standard error (mean of six experiments). S-1 samples were modified with DHNBS at a molar excess varying from 15 to 100. For conditions of the ATPase assays, see Materials and Methods. Control (100%) activities in μmol of P_i mg^{-1} min^{-1}: K^+(EDTA) activated, 4.2; Ca^{2+} activated, 0.65; actin activated, 0.52.

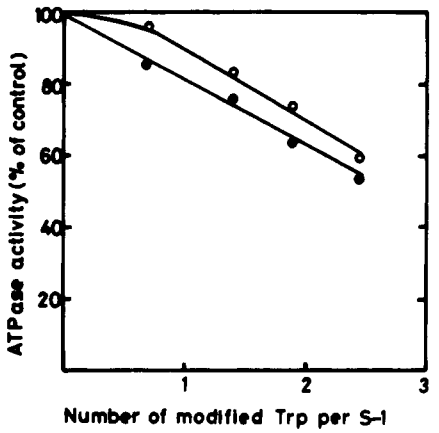

FIGURE 3: Effect of DHNBS modification on the ATPase activity of IAEDANS-labeled S-1. For conditions of modifications and ATPase assays, see Materials and Methods. A total of 1.3 mol of IAEDANS was introduced per mole of S-1 prior to the DHNBS treatment. S-1 samples were modified with DHNBS at molar excesses varying from 15 to 100. (○) K^+(EDTA)-activated and (●) Ca^{2+}-activated ATPase activities. Control (100%) activities in μmol of P_i mg^{-1} min^{-1}: K^+(EDTA) activated, 0.27; Ca^{2+} activated, 3.3.

specific fluorescence intensity (Figure 6).

The effect of MgATP on the tryptophan fluorescence was measured on HNB-modified S-1 and compared to that found for control S-1, $\Delta I/I = 15.5\%$ (Werber et al., 1972). In the range of 0.9–2.2 HNB groups per S-1, the intensity enhancement, ΔI, was found to remain unaltered (results not shown).

In order to find the class of Trps that is modified, we measured the acrylamide quenching of the intrinsic fluorescence of control and HNB-modified S-1. The results were analyzed according to the modified Stern–Volmer equation (Lehrer 1971): $I_0/\Delta I = 1/f_a + 1/f_a K_{sv}$ [Q], where I_0 is the fluorescence intensity of the unquenched sample, ΔI is the decrease in intensity upon addition of various concentrations of acrylamide, f_a is the fraction of the Trp residues, which is accessible to the fluorescence quencher, [Q] is its concentration, and K_{sv} is the Stern–Volmer constant. Plots of $I_0/\Delta I$ vs. 1/[Q] gave straight lines, from which the values of f_a and K_{sv} could be derived (Figure 7A). As the modification degree increases, i.e., as up to 2.2 HNB groups are incorporated in S-1, the degree of accessibility (f_a) of the Trps to the quencher, as derived from the modified Stern–Volmer plot, decreases (Figure 7B). On the other hand, the average Stern–Volmer constant, K_{sv}, does not seem to depend on the extent of modification and is 10.8 ± 0.5 M^{-1}.

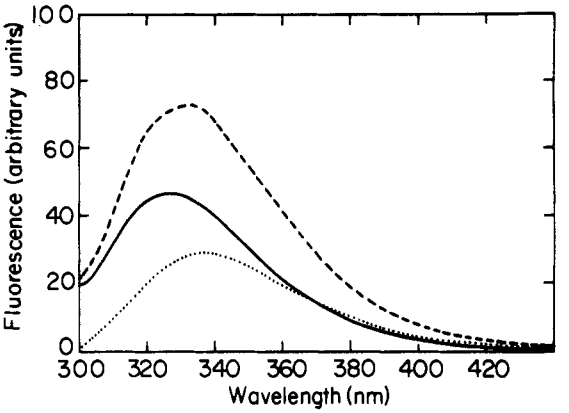

FIGURE 4: Fluorescence spectra of S-1 before and after modification with DHNBS. Performed on a SLM Model 8000 spectrofluorometer. Excitation wavelength was 295 nm at 25 ± 0.1 °C. (– –) Control S-1, 127 μg/mL; (—) S-1 modified at a 50-fold molar excess of DHNBS, 199 μg/mL. This spectrum was normalized with respect to the protein concentration of control S-1. (···) Difference spectrum between control and modified S-1.

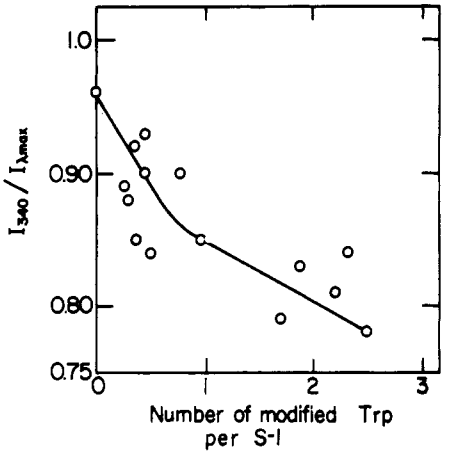

FIGURE 5: Dependence of the ratio between fluorescence intensities at two wavelengths on the number of modified Trps in S-1. Values taken from fluorescence spectra obtained under conditions similar to those of Figure 3. S-1 samples were modified with DHNBS at molar excesses varying from 15- to 150-fold.

In order to find the location of the most reactive tryptophan among the three heavy chain domains of S-1 (Balint et al., 1978; Mornet et al., 1981), we subjected the HNB-modified S-1 to limited trypsinolysis, dissociated the resulting fragments with GdmCl, and precipitated the 27-kDa and partially the 50-kDa fragments with ethanol. The heavy chain fragments were isolated from the precipitate (Figure 8A) and supernate (Figure 8B) by gel filtration chromatography. The first protein peak (I) in both panels A and B of Figure 8 contained the 50-kDa fragment, while peak II in panels A and B of Figure 8 contained the 27-kDa and the 20-kDa fragments, respectively, as indicated by $NaDodSO_4$–PAGE (not shown). The HNB and protein content of the eluted fractions were determined, and the extent of HNB modification per mole of fragment was estimated (Table II). The results show that,

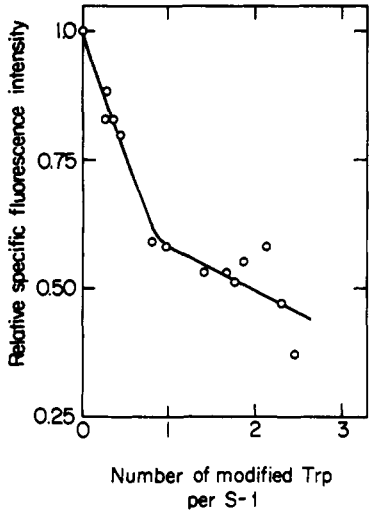

FIGURE 6: Effect of the modification (as expressed by the number of HNB groups incorporated in S-1) on the specific fluorescence intensity. Fluorescence intensities values taken from emission spectra obtained under the conditions of Figure 4. These values were then divided by the S-1 protein concentration. The resulting values were normalized with respect to unmodified S-1.

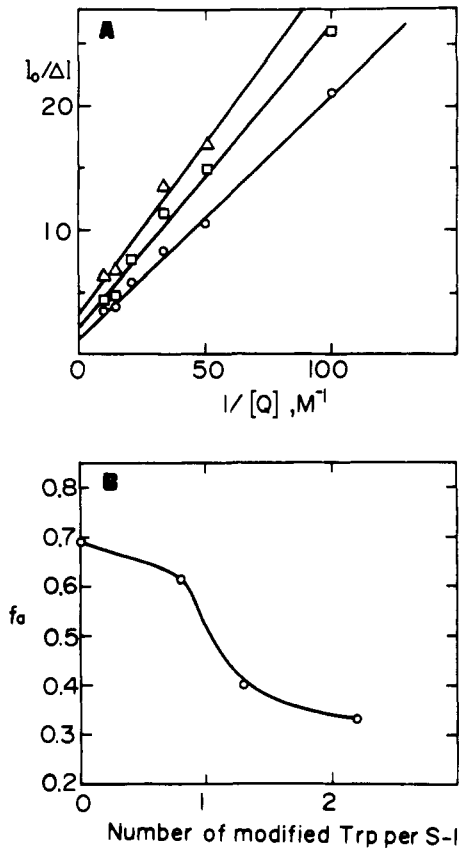

FIGURE 7: Acrylamide quenching of the fluorescence of control and HNB-S-1. (a) Modified Stern–Volmer plots, according to Lehrer (1971), from which the fraction of accessible Trps, f_a, is determined: (○) control S-1; (□) 1.3 HNB groups per S-1; (△) 2.2 HNB groups per S-1. (B) Dependence of f_a on the number of modified Trps per S-1.

under the conditions when about one Trp per S-1 was modified, more than 70% of the HNB label was incorporated to the 27-kDa fragment, indicating that the most reactive Trp is located in this domain.

Discussion

DHNBS is a reagent that in addition to its selectivity for

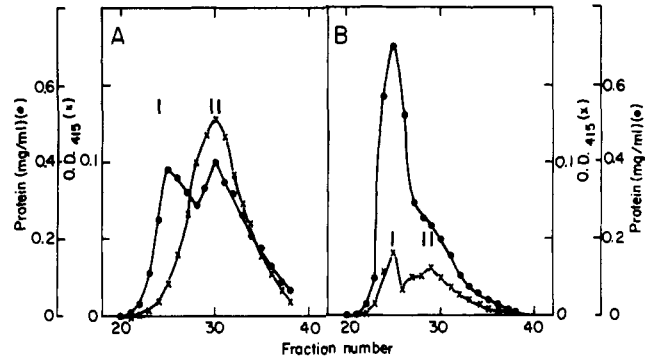

FIGURE 8: Separation of the major tryptic fragments of the S-1 heavy chain. S-1 was reacted with 15-fold molar excess of DHNBS and then digested with trypsin. The resulting tryptic fragments were dissociated by 6 M GdmCl. After ethanol treatment the precipitated 27-kDa fragment was separated from the 20-kDa fragment by centrifugation, while the 50-kDa fragment was evenly distributed between the precipitate and the supernate. Both the precipitate containing 50-kDa and 27-kDa fragments (A) and the supernate containing 50-kDa and 20-kDa fragments (B) were consecutively applied to a Sephadex G-100 column (60 × 2.6 cm) after solubilization in NaDodSO$_4$. Protein content (●) and OD at 415 nm (×) of the eluted fractions were measured, and the protein peaks were analyzed by NaDodSO$_4$–PAGE. In (A), peak I contained the 50-kDa fragment and peak II the 27-kDa fragment; in (B), peak I contained the 50-kDa fragment and peak II the 20-kDa fragment.

Table II: Distribution of HNB Groups between the Major Tryptic Fragments of the S-1 Heavy Chain

S-1 or its tryptic fragments[a]	mol of HNB/mol of S-1 or fragment	notes
S-1	2.16	before gel filtration
S-1	1.05	after gel filtration
27 kDa	0.71[b]	
50 kDa	0.13[b]	
20 kDa	0.10[b]	

[a] At 15-fold molar excess of DHNBS over S-1. [b] Estimated from the results of the gel filtration chromatography presented in Figure 8.

Trp possesses several advantages over other tryptophan modification reagents: it reacts readily under mild—unharmful to S-1—conditions (temperature and pH) and provides stable covalent attachment of the environmentally sensitive HNB chromophore (Horton & Tucker, 1970). It has to be stressed, however, that precautions have to be taken in order to ensure that the noncovalent-bound molecules of the reagent are separated from the labeled protein: under our conditions exhaustive dialysis was not sufficient, and we had to perform an additional gel filtration step.

The mildness of the modification conditions is substantiated by the magnitude of the changes in the ATPase activities: the actin-activated ATPase is not affected significantly throughout the whole range up to 2.5 modified Trps, and neither Ca^{2+}- nor K^+(EDTA)-activated ATPase change by more than 20% when the first Trp is modified. The observed effect of DHNBS modification of S-1 ATPase activities, increase in Ca^{2+}-activated and decrease in K^+(EDTA)-activated ATPase activity (Figure 2), is similar to the change obtained upon SH_1 labeling (Sekine & Yamaguchi, 1963). This might indicate that the ATPase changes are not due to Trp but to a partial SH_1 modification. The finding that DHNBS treatment affects both ATPase activities, even when the SH_1 thiol is specifically blocked by IAEDANS (Takashi et al., 1976), indicates that Trp modification has a direct influence on the ATPase activity of S-1 (Figure 3). However, one cannot exclude the possibility of a partial modification of unblocked SH_1 by DHNBS, also contributing to the observed changes in ATPase activities.

The number of HNB groups incorporated increases up to a value of 2.5–3.0, with increasing molar excess of DHNBS (Figure 1). There is a parallel rise in the number of modified Trps, as determined from the fluorescence of the denatured protein in 6 M GdmCl, a method based on the observation that the fluorescence of HNB-modified tryptophan residues in proteins is quenched (Naik & Horton, 1973). The agreement between the number of HNB groups incorporated and the number of Trps modified indicates that HNB incorporation occurs essentially in Trps and only at a substitution extent of one HNB per Trp.

With modified S-1 samples having 0.9–2.2 HNB residues per S-1, no significant change was observed in the fluorescence effect caused by the addition of MgATP.[3] This implies that the Trps whose fluorescence is increased by nucleotide binding and hydrolysis, and which are, according to Torgerson's work (1984), mainly those having an intermediate lifetime, are not being modified by DHNBS.

As Trps are being modified by DHNBS the "specific fluorescence intensity" (which is equivalent to quantum yield) drops gradually to less than 50%. The emission spectrum of HNB-S-1 is blue-shifted with respect to that of native S-1. This indicates that the contribution of Trps, whose emission is in the red part of the spectrum, namely, those located in a polar environment, is missing (Elkana, 1968). This is a result of the total quenching of the fluorescence of HNB-modified Trps (Naik & Horton, 1973). Thus, those Trps that are located in a polar environment are modified by DHNBS, or else, those whose environment is hydrophobic are not affected. According to Torgerson (1984) the long-lifetime Trps emit in the red, and therefore, it is probable that these are the ones modified by DHNBS.

With respect to acrylamide quenching we found that at 25 °C 70% of the Trp emission is quenchable (f_a) in native S-1, whereas this fraction decreases gradually to 33% after modification of 2.5 Trps. The quenching constant K_{sv} remains practically unchanged and within error the same as that obtained by Torgerson (1984) at 4 °C, whereas we find a much larger f_a for nonmodified S-1 than his (50%), probably due to different temperatures. All the fluorescence results agree well with our premise above that the Trps which became modified by DHNBS are the ones located in the more polar environment. These results are also in agreement with those of Torgerson (1984), who concluded that acrylamide affects only the long-lifetime component whose spectrum peaks at 345 nm ("exposed Trp"), whereas ATP affects the Trp class with intermediate lifetime whose spectrum peaks at 330 nm ("buried Trp"). Thus we observe three main classes of Trps in S-1: a first class of Trp can be modified by DHNBS, is quenchable by acrylamide, consists of exposed residues whose emission is in the red part of the spectrum, and can probably be associated with a long lifetime; a second class of Trp(s) is affected by ATP, is nonmodifiable by DHNBS, is nonquenchable by acrylamide, and therefore consists of buried residues and can probably be associated with an intermediate lifetime; finally, a third class of Trp(s) is quenched by acrylamide but is nonmodifiable by DHNBS. This last class was observed after modification of 2.5 Trps, since it was found that 33% of the nonmodified Trps remained quenchable by acrylamide after modification with DHNBS. The nature of this class is not known, and it is possible that the Trp(s) of this class become(s) accessible to acrylamide only after modification, which might affect the structural integrity of the S-1 molecule. It is difficult to assign this class with any of Torgerson's lifetime components.

The major site of HNB labeling when a single Trp is modified is the 27-kDa fragment of S-1, which contains according to the published sequence (Tong & Elzinga, 1983) two Trps, Trp-112 and Trp-130. The latter Trp was shown to react with a photosensitive ATP analogue (Okamoto & Yount, 1985). One might assume that both Trps of the 27-kDa fragment are exposed, whereas those of the 50-kDa Trps could be unexposed. This is supported but not proved by the fact that we could not find any long-lifetime and acrylamide-accessible Trp in the isolated 50-kDa fragment (Muhlrad et al., 1986). If our assignment into the above classes is correct, it would seem that ATP enhances the fluorescence of one or more Trps that are located in the 50-kDa fragment.

It should be mentioned that Yoshino et al. (1972) and Yoshino (1976) modified HMM with 2-hydroxy-5-nitrobenzyl bromide, a reagent that is similar to but not identical with DHNBS, and found two HNB bound at a 100-fold molar excess of reagent over HMM. One of the two HNB was bound to the S-2 segment and the other to one of the two S-1 segments of the HMM. In other words, the maximal number of HNB bound per S-1 was 0.5, which is far less than that we have found in this study. The discrepancy between Yoshino's and our results could be due to either the use of different reagents or the fact that in Yoshino's studies HMM was modified instead of S-1.

In conclusion, we found with the help of the specific DHNBS modification that S-1 Trps could be classified into accessible and nonaccessible classes with well-characterized spectral properties. Most of the accessible Trps are located in the 27-kDa fragment, while those Trps that are nonaccessible and whose fluorescence is perturbed by ATP–Trp(s) probably reside on the 50-kDa domain of the S-1 segment.

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[3] The previously reported reduction in the magnitude of the effect of ATP on Trp intrinsic fluorescence (Werber & Muhlrad, 1985) was based on data obtained with modified S-1, from which not all the noncovalently bound reagent molecules had been removed.

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The Cytochrome Subunit of the Photosynthetic Reaction Center from *Rhodopseudomonas viridis* Is a Lipoprotein†

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ABSTRACT: Using automated procedures for Edman degradation and for the identification of the derived phenylthiohydantoin–amino acids of the cytochrome subunit in the photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis*, the phenylthiohydantoin derivative of the first amino acid could not be detected. However, the N-terminus of the cytochrome subunit was not blocked, and a phenylthiohydantoin derivative could be isolated after manual Edman degradation. It contained two kinds of covalently bound fatty acids (18:OH and 18:1); the pattern of molecular species obtained by reversed-phase high-performance liquid chromatography and the specific fatty acid composition of the separated species were only consistent with two ester bonds per molecule. Mass spectroscopic analysis provided evidence that the N-terminal amino acid was a cysteine which was linked to a diglyceride via a thioether bond, as has been first described for the N-terminus of the major outer membrane lipoprotein from *Escherichia coli* [Hantke, K., & Braun, V. (1973) *Eur. J. Biochem. 34*, 284–296]. However, the cytochrome subunit lacked the acylation by a fatty acid at the N-terminal amino group. In addition, the DNA coded for a cysteine as the N-terminal amino acid and for a preceding peptide sequence characteristic for signal sequences of bacterial lipoproteins. The fatty acids seem to anchor the cytochrome subunit in the photosynthetic membrane which is an invagination of the inner bacterial membrane.

Photosynthetic reaction centers are complexes of pigments and integral membrane proteins which catalyze the primary steps in photosynthesis. Upon absorption of light, an electron is transferred across the photosynthetic membrane [for a review, see Okamura et al. (1982)]. Recently, the photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis* was crystallized (Michel, 1982). The subsequent X-ray structure analysis allowed the calculation of an electron density map at 3-Å resolution. As a first result, the arrangement of the photosynthetic pigments was presented (Deisenhofer et al., 1984). For a reliable interpretation of the protein part, the amino acid sequences of the four protein subunits (H, M, L, and a cytochrome of the *c* type) were needed. The amino acid sequences of the H, M, and L subunits were elucidated by sequencing the DNA coding for these subunits and by chemically sequencing the isolated protein subunits and fragments therefrom (Michel et al., 1985, 1986). The amino acid sequence of the cytochrome subunit has now been completely determined (K. A. Weyer et al., unpublished results). During the protein sequence work on the cytochrome subunit, the unusual behavior of the N-terminal amino acid led us to further investigate its structure. In this paper, we provide evidence that the N-terminal amino acid is a cysteine linked to a diglyceride via a thioether bond as has been described for

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