Coupling of Dyes to Biopolymers by Sensitized Photooxidation. Affinity Labeling of a Binding Site in Bovine Serum Albumin†

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ABSTRACT: Sensitized photooxidation is normally used to destroy or modify certain amino acid residues in proteins. Using high concentrations of dye it was found possible, however, to couple dyes to various macromolecules. Irradiation of air-saturated solutions of bovine serum albumin–fluorescein, bovine serum albumin–Acridine Orange, bovine serum albumin–Methylene Blue, ribonuclease–fluorescein, ribonucleic acid–fluorescein, and dextran–fluorescein with visible light resulted in coupling of dye to the macromolecule. The degree of coupling obtained was generally about 0.5 mg of dye/g of macromolecule. Further studies of the bovine serum albumin–fluorescein and ribonuclease–fluorescein systems showed that the quantum yields for coupling were about 10^{-4} compared to 10^{-3}–5×10^{-3} for inactivation, aggregation, and amino acid destruction. Coupling also occurred in oxygen-free solutions but with considerably lower yields. Coupling yields increased strongly with increase in the light intensity. The possible specificity of the coupling was investigated in the bovine serum albumin–fluorescein system since bovine serum albumin is known to contain binding sites having affinity for fluorescein. Tryptic digestion of the albumin–fluorescein adduct followed by separation of the peptides mainly yielded one labeled tripeptide. The sequence was found to be X-Leu-Tyr where X is the residue containing the fluorescein label. A comparison with the known parts of the bovine serum albumin sequence indicates that it is identical with a sequence Tyr-Leu-Tyr found about 130 residues from the N-terminal end close to one of the two tryptophans in the protein. It is possible that photoinduced coupling can be used as a general method for affinity labeling.

Dye-sensitized photochemical oxidation of enzymes has often been used to obtain information on the amino acid residues essential for the catalytic activity of an enzyme. Selective modification of histidine, tryptophan, methionine, and tyrosine residues can generally be achieved. A subsequent loss of activity indicates that one or several of these residues participates in the catalytic process or is essential for maintaining the native conformation of the enzyme. By using different types of sensitizers and/or various conditions during irradiation it has been possible to get an even more selective action of the photooxidation. This is beautifully exemplified in the series of studies by Jori *et al.* (1968a,b,1969) in which methionine has been selectively oxidized leaving histidine, tryptophan, and tyrosine unaffected. A recent further development in using photooxidation as a tool in protein chemistry has been to couple the sensitizer to some reactive group in the protein and then modify certain groups in the neighborhood of the coupled sensitizer (Scoffone *et al.*, 1970).

The techniques mentioned above all result in chemical modification of one or several amino acid residues such as the oxidation of methionine to methionine sulfoxide or tryptophan to kynurenine. This type of alteration is usually rather difficult to localize in a normally sized protein because the changes involved are comparatively small resulting in difficult detection and separation problems in the degradation and peptide studies necessary for localizing the modified residues. This problem would be considerably easier to solve if modification had involved the coupling of some suitable substance to the amino acid residue(s) affected. However, under the conditions normally used for dye-sensitized photooxidation no coupling takes place and only destruction or modification of certain amino acid residues occurs.

In this study the possibility of inducing coupling of dyes to various biopolymers has been investigated. It was found that upon increasing the concentration of dye above that normally used coupling occurs. Several different dyes could be coupled to various biopolymers. It was also found that the coupling could in certain cases be very specific and that the method probably can be used for affinity labeling of binding sites.

Materials and Methods

The bovine serum albumin used was obtained from Statens Bakteriologiska Laboratorium (Stockholm). It contained about 7–8% dimer and polymer. Pure monomer was prepared by gel filtration on Sephadex G-150. The fatty acid content of the monomer as determined by gas chromatography (Hagenfeldt, 1966) was 0.73 ± 0.05 mol of fatty acid/mol of albumin. The molecular weight of monomeric bovine serum albumin was assumed to be 66,000.

Trypsin (DCC-treated), ribonuclease A, and RNA were obtained from Sigma Chemical Co. Carboxypeptidase A was purchased from Koch Light.

Fluorescein (G. T. Gurr, London) was purified by precipitation from 0.01 M NaOH solution with acetic acid. The procedure was repeated three times. The molar extinction coefficient of the purified fluorescein in 0.01 M NaOH was 8.4×10^4 M^{-1} cm^{-1} at 491 nm. All other compounds were of reagent grade or of the highest grade commercially available.

Illumination Procedures. A high-pressure mercury lamp

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(Gates 420-U2, 250 W) was used for all irradiation experiments. Unless stated otherwise, all samples have been illuminated in an open flat-bottomed beaker for 30 min at a distance of 20 cm from the irradiation source. At this distance the intensity was 1.5×10^{16} photons sec^{-1} cm^{-2} using the nitrite filter described below and 3.4×10^{16} photons sec^{-1} cm^{-2} using a glass filter. The temperature was maintained at 20° by circulating water. The thickness of the sample layer was 0.2–0.5 cm and the solutions were stirred continuously by a stream of air or a magnetic stirrer. In all experiments a glass beaker with water was used to absorb the wavelengths below 320 nm and the infrared irradiation. In most experiments a 2-cm filter solution of 0.5 M sodium nitrite was used in addition to the glass filter to absorb the wavelengths below 400 nm. The intensity was determined using the ferrioxalate actinometer (Parker, 1953; Lee and Seliger, 1964).

Determination of the Degree of Coupling. The dye concentration was determined by measuring the visible absorption at the wavelength of maximum absorption after separation of excess dye by gel filtration. It was assumed that the extinction coefficient was the same for free and coupled dye. A known volume of the sample was then lyophilized and dried. The degree of coupling was calculated on the basis of the dry weight of the sample.

Chromatographic Procedures. All gel filtration experiments were performed on columns of Sephadex gel. Unless otherwise specified, the columns have been equilibrated and eluted with 0.1 M ammonia–ammonium acetate buffer (pH 9.2). SE-Sephadex ion exchange chromatography was performed on a 1.6 × 40 cm column. The gel was washed with 0.1 M NaOH and 0.1 M HCl prior to equilibration with 0.1 M acetic acid–0.1 M formic acid buffer adjusted to pH 3.0 with ammonia. The pH gradient elution was accomplished by attaching the column to a 400-ml reservoir of the 0.1 M acetic acid–0.1 M formic acid buffer to which 0.4 M ammonia solution was added continuously.

Tryptic Digestion. Digestion was started by adding 1.5 mg of trypsin/100 mg of protein to a 7% solution of the protein in 0.1 M sodium phosphate buffer (pH 7.9). Three further additions of the same amount of trypsin were made 1, 2, and 4 hr later. The pH of the solution was maintained between 7.7 and 7.9 by addition of 1 M NaOH. To avoid disulfide exchange reactions and bacterial growth, 0.5 ml of 0.01 M *N*-ethylmaleimide solution was added. The hydrolysis was allowed to proceed for 8 hr at 37° and the reaction mixture was then allowed to stand at room temperature overnight.

Peptide Degradation with Carboxypeptidase A. The peptide material (100 nmol) was dissolved in 100 μl of 0.05 M sodium bicarbonate buffer (pH 8.3); 10 μg of carboxypeptidase A in 10 μl of 0.01 M sodium bicarbonate buffer (pH 10.0) was added and the mixture was incubated for 2 hr at 37°.

Amino Acid Analysis. The samples were hydrolyzed in sealed and evacuated Pyrex tubes for 24 hr with 6 M HCl at 110°. Analyses were performed with a fully automatic Bio-Cal BC 200 amino acid analyzer.

Activity Determinations of Ribonuclease. The spectrophotometric method of Kunitz (1946) was used with RNA as substrate. Sample solutions (2 ml) were mixed with 2 ml of 0.1% RNA in 0.1 M ammonium acetate buffer (pH 5.0) and the initial decrease in absorption at 300 nm *vs.* time was measured.

Spectrophotometric measurements were made with a Zeiss PMQ II spectrophotometer.

Quantitative Measurement of Amino Groups. The material was dissolved in water and lyophilized after addition of a small amount of 1 M NaOH to give pH 10–11. This procedure was repeated three times to remove ammonia. Determination of amino groups was then performed by using trinitrobenzenesulfonic acid in the presence of sulfite according to Fields (1971).

Determination of N-Terminal Amino Acid. N-terminal amino acid analyses were performed using the dansylation procedure essentially as described by Gray (1967). About 25 nmol of peptide was dissolved in 50 μl of 0.2 M sodium bicarbonate buffer (pH 8.5) and 50 μl of 1-dimethylaminonaphthalene-5-sulfonyl chloride (5 mg/ml of acetone) was added. After incubation for 1 hr at 37° the mixture was dried *in vacuo*. Identification of dansyl amino acids was performed using thin-layer chromatography on polyamide plates in benzene–glacial acetic acid according to Woods and Wang (1967).

Results

The concentrations of dye usually used in experiments with dye-sensitized photooxidation are in the range 10^{-5}–10^{-4} M. To study the possibility of coupling dye to the protein instead of just getting modification and destruction of amino acid residues, a series of experiments was performed where the concentration of dye was varied. The system chosen for this study was bovine serum albumin–fluorescein. Various solutions containing 1% of monomeric albumin in 0.02 M sodium phosphate buffer (pH 8.0) were illuminated using a nitrite filter. After illumination the samples were subjected to gel filtration on Sephadex G-150, allowing both determination of degree of coupling as described in Materials and Methods and the degree of aggregation of albumin. Table I shows the results obtained. It is evident that the concentration of dye strongly affects the degree of coupling. In 10^{-5} M fluorescein little coupling takes place as compared to that obtained in a 5×10^{-3} M solution. Aggregation of the albumin is induced by the treatment and is about the same at all concentrations of dye.

The coupling is not a special feature of the bovine serum albumin-fluorescein system. Studies on other systems showed that coupling of dye could be accomplished in all cases investigated. Table II shows the results obtained with some different systems. The various systems contained 1% macromolecule and 1 mM dye in 0.02 M sodium phosphate buffer (pH 8.0) and illumination was performed using a nitrite filter. The degree of coupling was determined after

TABLE I: Coupling of Fluorescein to Bovine Serum Albumin and Aggregation of the Albumin upon Illumination of Samples of 1% Albumin in 0.02 M Sodium Phosphate Buffer (pH 8.0) Containing Various Concentrations of Fluorescein.

Concn of Fluorescein (mM)	Degree of Coupling, mol of Fluorescein/mol of Albumin	Aggregates in Per Cent of the Total Amount of Albumin
5.0	0.120	12
1.0	0.095	12
0.10	0.014	11
0.010	<0.003	9

TABLE II: Coupling of Dye to Macromolecule upon Illumination of Various Systems of 1 mM Dye and 1% Macromolecule in 0.02 M Sodium Phosphate Buffer (pH 8.0).

System	Degree of Coupling: mol of Dye/mol of Macromolecule	Degree of Coupling: mg of Dye/g of Macromolecule
Ribonuclease–fluorescein	0.021	0.51
Bovine serum albumin–fluorescein	0.095	0.48
Dextran–fluorescein	0.016	0.18
RNA–fluorescein	1.2	0.40
Bovine serum albumin–Acridine Orange	0.13	0.62
Bovine serum albumin–Methylene Blue	0.060	0.29

separation of excess dye by gel filtration on Sephadex G-25. Two of the systems, bovine serum albumin–fluorescein and ribonuclease–fluorescein, were chosen for a more detailed study.

Studies on the Ribonuclease–Fluorescein System. Three samples of 1.0% ribonuclease solutions in 0.02 M phosphate buffer (pH 8.0) containing 0.1, 0.3, and 1.0 mM fluorescein were illuminated using a nitrite filter. Aliquots of samples were subjected to gel filtration on a Sephadex G-50 column and eluted with 0.1 M acetic acid–ammonium acetate buffer (pH 4.7). The elution curves obtained are shown in Figure 1. It is evident that the degree of aggregation increases with increasing concentration of fluorescein. The unfractionated samples and the aggregate and monomer fractions from gel filtration were studied with respect to degree of coupling, enzymatic activity and amino acid composition. The quantum yields were calculated for the unfractionated samples and the values obtained are given in Table III. It is evident that coupling is a very minor reaction compared with the other processes. As with bovine serum albumin, it is strongly dependent on the concentration of the fluorescein which is not the case with the other processes. The quantum yields for inactivation and amino acid destruction are similar to those previously obtained in other systems. The amino acids affected were mainly histidine, methionine, cystine, and tyrosine. The quantum yields for amino

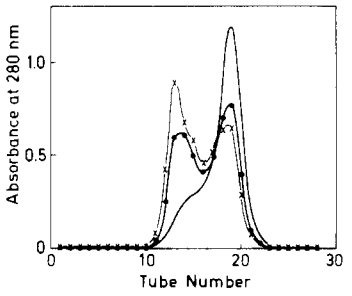

FIGURE 1: Elution diagram of a Sephadex G-50 gel filtration (column 2.0 × 80 cm) of 1% ribonuclease samples that had been illuminated in the presence of various concentrations of fluorescein. Elution patterns: (—) 0.1 mM fluorescein; (●) 0.3 mM fluorescein; (x) 1 mM fluorescein.

TABLE III: Quantum Yields for Various Processes upon Sensitized Photooxidation of 1% Ribonuclease Solutions in 0.02 M Sodium Phosphate Buffer (pH 8.0) Containing Various Concentrations of Fluorescein.[a]

Concn of Fluorescein (mM)	Quantum Yields (× 10^4): Coupling	Aggregation	Inactivation	Amino Acid Destruction
1.0	0.84	10	8.0	34
0.30	0.26	7.8	7.2	32
0.10	0.05	2.8	6.0	27

[a] The values given are the total quantum yields of the sample.

acid destruction were calculated from the sum of the decreases of the individual amino acids using aspartic acid, valine, and leucine as nonchanging standards. The largest decrease observed was 23% for tyrosine.

The results obtained from the studies on the fractions from gel filtration showed that there were considerable differences between the aggregate fractions and the monomer fractions. The degree of coupling was generally about three times higher in the aggregate fractions and the enzymatic activity between 30 and 50% of that of the monomer fraction. The activities of the monomeric fractions were about 80–90% of the activity of native ribonuclease. The degree of amino acid destruction was 20–30% higher in the aggregate fractions. A comparison of the values obtained with the gel filtration fractions and the corresponding unfractionated samples, taking into account the various compositions, showed good agreement.

The aggregate fraction from gel filtration of the ribonuclease sample illuminated in the presence of 1 mM fluorescein was studied with regard to stability using gel filtration. The aggregates did not dissociate appreciably upon standing and repeated gel filtration. However, reduction with 0.1 M mercaptoethanol in 0.05 M Tris buffer (pH 8.5) for 0.5 hr converted 35% of the material to monomer. This is interpreted as an indication that aggregation induced by the sensitized photooxidation proceeds to an important degree by formation of intermolecular disulfide bonds.

Studies on the Bovine Serum Albumin–Fluorescein System. It was shown in Table I that the coupling of fluorescein to bovine serum albumin is strongly dependent on the concentration of fluorescein. It was also found that sensitized photooxidation caused aggregation of the albumin and that this is independent of the fluorescein concentration. Data on amino acid destruction was obtained in an experiment where a 1.0% bovine serum albumin solution containing 1 mM fluorescein in 0.02 M sodium phosphate buffer (pH 8.0) was illuminated for 60 min using a nitrite filter. The degree of coupling was 0.20 mol of fluorescein/mol of albumin. Amino acid analysis, performed after gel filtration on Sephadex G-25, showed that the following amino acids had decreased: histidine 15% (17), cystine 12% (17), methionine 35% (4), and tyrosine 23% (19). The numbers of residues in native bovine serum albumin (King and Spencer, 1970) are given in parentheses. Fractionation of an aliquot of the sample on Sephadex G-150 showed the presence of

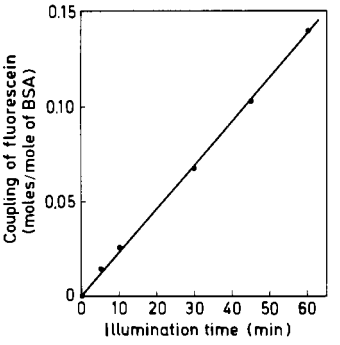

FIGURE 2: Kinetics for the coupling of fluorescein to bovine serum albumin upon illumination of a 1% solution in 0.02 M sodium phosphate buffer (pH 8.0) containing 1 mM fluorescein.

11% aggregates which were mostly dimeric. Amino acid analysis of the monomer and aggregate fractions showed 20–30% higher amino acid destruction in the aggregate fraction than in the monomer fraction. This is in close agreement with that found for ribonuclease.

The bovine serum albumin–fluorescein system was chosen for further studies on the factors influencing coupling. Illumination was performed using a nitrite filter on a solution of 1% albumin containing 1 mM fluorescein in 0.02 M sodium phosphate buffer (pH 8.0). The kinetics of the coupling were followed by removing aliquots of the solution at various times and measuring the degree of coupling after separation of free fluorescein by gel filtration on Sephadex G-25. The results obtained are shown in Figure 2 which demonstrates that there is a linear relationship between the degree of coupling and the duration of illumination. This is expected since neither the dye concentration nor the amount of reactive groups in the protein changes appreciably during the illumination times used.

The effect of illumination on the degree of coupling was studied at different wavelengths. Two samples of 1% albumin in 0.02 M sodium phosphate buffer (pH 8.0) containing 1 mM fluorescein were illuminated using different filters. The coupling yields were 0.152 and 0.095 mol of fluorescein/mol of albumin using a glass filter and a nitrite filter, respectively. Taking account of the known intensity distribution of the mercury lamp, it could be calculated that the illumination at 436 nm was 2.1 times more efficient than at 365 nm in inducing coupling. The absorption of the fluorescein is higher at 436 nm than at 365 nm but the concentration of dye is so high that even at 365 nm more than 90% of the light is absorbed by the solution. However, the energy of the 436-nm light is absorbed in a smaller volume which results in a higher local concentration of excited molecules and could possibly facilitate higher coupling yields.

The relationship between yield of coupling and light intensity was studied in a series of experiments in which the distance between the lamp and the sample was varied. The illumination dose was 1.0×10^{19} photons cm^{-2} in all cases. Illumination was performed using a glass filter on a solution of 1% albumin containing 1 mM fluorescein in 0.02 M sodium phosphate buffer (pH 8.0). Table IV shows the results obtained. It is evident that the degree of coupling is strongly dependent on the light intensity, supporting the suggestion made above that an increased local concentration of excited molecules results in higher coupling yields.

Dye-sensitized photooxidation is usually dependent on the presence of oxygen. A number of experiments were performed where 1.0% albumin solutions containing 1 mM fluorescein in 0.02 M sodium phosphate buffer (pH 8.0) were illuminated in closed glass tubes in equilibrium with air and after deoxygenation, respectively. Deoxygenation was accomplished by allowing a stream of oxygen-free argon to pass through the solution for 2 hr. The samples were illuminated for 10 min at a distance of 10 cm from the illumination source. The air-saturated sample showed a degree of coupling of 0.097 mol of fluorescein/mol of albumin. The degree of coupling for the deoxygenated sample was 0.034 mol of fluorescein/mol of albumin.

TABLE IV: Quantum Yields at Various Intensities for the Coupling of Fluorescein to Bovine Serum Albumin.[a]

Intensity ($\times 10^{-16}$) (photons sec^{-1} cm^{-2})	Quantum yields ($\times 10^5$)
16	30
7.7	16
3.4	7.9
1.4	3.9

[a] The samples were 1% in albumin and 1 mM in fluorescein. The dose given was the same for all samples.

Prevention of coupling could be accomplished by addition of cysteine. A 1.0% albumin solution containing 1.0 mM fluorescein in 0.02 M sodium phosphate buffer (pH 8.0) was made 5 mM with respect to cysteine. The solution was illuminated for 20 min at a distance of 10 cm using the glass filter and in equilibrium with air. Determination of the degree of coupling after gel filtration of an aliquot of the solution on Sephadex G-25 yielded a value of less than 0.003 mol of fluorescein/mol of albumin. The presence of cysteine evidently inhibits the coupling.

Specificity of the Coupling in the Bovine Serum Albumin–Fluorescein System. For studies on the possible specificity of the photoinduced coupling of dyes it was decided to use the bovine serum albumin–fluorescein system. This system has several advantages. The photochemistry of fluorescein is fairly well known. Most important, however, is the fact that fluorescein binds to albumin. Bovine serum albumin contains three weak binding sites for fluorescein at neutral and weakly alkaline pH. The sequence around one of these binding sites has been determined (Andersson *et al.*, 1971b). If there is specificity in the coupling reaction it may be specific in several different ways. One possibility is that it is specific for certain amino acid residues as, for instance, tyrosine or histidine. Another and more interesting possibility is that if there is a binding site for the dye one may also get preferential coupling to this site. The bovine serum albumin–fluorescein system allows the investigation of both these possibilities.

About 60 ml of a 1.0% albumin solution containing 1 mM fluorescein in 0.02 M phosphate buffer (pH 8.0) was illuminated for 60 min using a nitrite filter. Excess fluorescein was then removed by gel filtration on Sephadex G-25. The degree of coupling was 0.18 mol of fluorescein/mol of albumin. The albumin fraction was concentrated by ultrafiltration and digested with 36 mg of trypsin. The digestion mixture was subjected to gel filtration on a Sephadex G-50 column. The elution curve obtained is shown in Figure 3. It is evident that both high and low molecular weight fluorescein-labeled peptides are present. Fractions I and II were collected for further fractionation by ion-exchange chroma-

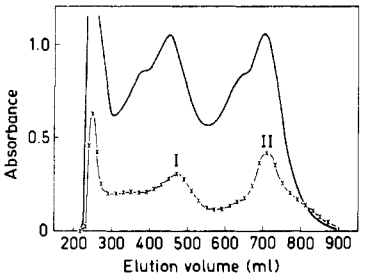

FIGURE 3: Elution diagram of a Sephadex G-50 gel filtration (column 3.2 × 90 cm) of a tryptic digest of a bovine serum albumin sample containing 0.18 mol of coupled fluorescein/mol of albumin. Elution patterns: (—) absorbance at 280 nm; (x) absorbance at 502 nm.

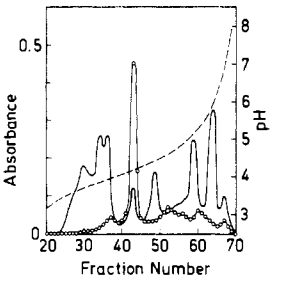

FIGURE 4: SE-Sephadex chromatography elution diagram of fraction II from gel filtration of a tryptic digest of a bovine serum albumin sample containing 0.18 mol of coupled fluorescein/mol of albumin. (- - -) pH. Absorbance was measured at 254 nm (—) and at 502 nm (O) after addition of 100 μl of 25% ammonia to give pH ≥9.

tography on SE-Sephadex using pH-gradient elution. The run on fraction I showed a large number of peaks, some of which were considerably more labeled with fluorescein than the others. As it was known from earlier studies (Andersson *et al.*, 1971a) that this fraction is a very complicated mixture of large peptides in the molecular weight range 8000–13,000 and heavily cross-linked by disulfide bonds, it was decided to stop further fractionation work on this peptide fraction. The low molecular weight peptide fraction, fraction II, contains the main part of the fluorescein label and the efforts were concentrated on this fraction. Ion-exchange chromatography on SE-Sephadex using pH-gradient elution gave the elution curve in Figure 4. One main peptide peak contained about 35% of the total fluorescein label of the fraction. This peptide was further purified by gel filtration on Sephadex G-25 and it appeared at 1.35 times the total volume of the column (V_t). The retardation of the peptide on Sephadex G-25 depends mainly on the adsorption of fluorescein to the gel. This results in a very valuable purification effect. The fluorescein-labeled small peptides elute considerably later than the corresponding unlabeled ones. A paper electrophoresis run of a small amount of the peptide indicated that it was pure. Only one fluorescein-labeled spot appeared that stained with ninhydrin. No other ninhydrin positive spots were found.

Amino acid analysis after acid hydrolysis on 56 nmol of peptide, as estimated from the visible absorption of the fluorescein using 8.4×10^4 M^{-1} cm^{-1} as the extinction coefficient at the absorption maximum, yielded 56 nmol of leucine and 54 nmol of tyrosine and no other amino acids. This result may be interpreted in two ways. If one assumes that the fluorescein amino acid adduct is cleaved during hydrolysis with formation of the original amino acid, then the fluorescein-labeled peptide must be a dipeptide. However, if the fluorescein amino acid adduct is stable to acid hydrolysis or is decomposed giving other products than the original amino acid then it must be a tripeptide containing leucine, tyrosine, and another amino acid.

N-terminal labeling on 25 nmol of peptide using dansylation followed by hydrolysis with 6 M HCl at 110° for 10 hr and thin-layer chromatography showed neither leucine nor tyrosine. The fluorescein spot did not move from the origin. This indicates that the peptide is a tripeptide and that the N-terminal amino acid is the one that had formed the adduct with fluorescein. Digestion of 100 nmol of peptide with 10 μg of carboxypeptidase A was performed and the digestion mixture was passed through a Sephadex G-25 column. The elution curve obtained showed three peaks at 254 nm. One uncolored peak with the same spectrum as tyrosine appeared at 1.22 V_t. Two colored peaks, one minor appearing at 1.62 V_t and one major at 2.11 V_t, were obtained. Free fluorescein appears at 2.9 V_t and the undigested peptide at 1.35 V_t in the system used. Amino acid analysis of the 1.62 V_t peak showed only one amino acid, leucine. The 2.11 V_t peak contained no ordinary amino acid at all. Dansylation of the material eluted at the total volume followed by thin-layer chromatography showed one spot with the same R_F value as leucine. Together these data only allow one sequence, namely X(F)-Leu-Tyr where X is the amino acid residue containing the coupled fluorescein (F). The 1.62 V_t peak is the dipeptide X(F)-Leu and the 2.11 peak the amino acid–fluorescein adduct X–F. It is well known that carboxy-terminal tyrosine is rapidly split off by carboxypeptidase A and this is in good agreement with the results obtained. It is further known that trypsin can split at certain tyrosine residues especially upon extensive degradation. A tryptic split at leucine is unlikely. Finally it should be pointed out that there is a direct relationship between elution volume on Sephadex G-25 and percentage of fluorescein in the molecule. The elution order is as follows, tripeptide, dipeptide, X–F adduct, and fluorescein.

About 90% of the sequence of bovine serum albumin has been determined (Brown *et al.*, 1971) and a search for Leu-Tyr sequences reveals two such sequences present. One of these is the Lys-Tyr-Leu-Tyr sequence situated about 130 amino acid residues from the N-terminal end and the other is the Ala-Ala-Leu-Tyr sequence about 290 residues from the N-terminal. The first of these two sequences fulfills all the necessary conditions to explain the formation of the labeled tripeptide. The lysine residue at the N-terminal side fits well with the tryptic split. The tyrosine residue following should be the labeled residue which is not unexpected since tyrosine is a sensitive amino acid in photosensitized reactions. The second sequence does not fulfill any of these conditions. Trypsin does not split at alanine and alanine is also one of the most inert amino acid in photosensitized reactions. However, as there are parts of the sequence of bovine serum albumin which are still unknown, one cannot be certain that the first sequence is that giving the labeled tripeptide.

In order to obtain further information about the nature of the X–F adduct some studies were performed on the X–F adduct prepared by the carboxypeptidase digestion of the tripeptide. As studies on visible and uv spectra and their pH dependence could be expected to give valuable information, the spectrum of the X–F adduct was determined at a number of pH values in the range of 3–12. The visible spectrum was the same as that of free fluorescein at all pH values studied with the exception that the maximum at neutral and alkaline pH was at 499 nm instead of at 491 nm. The changes in the fluorescein spectra upon ionization of the

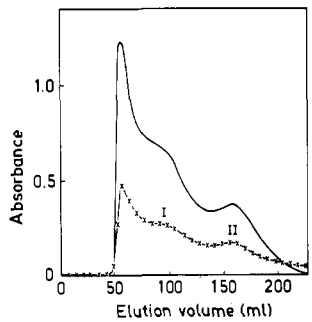

FIGURE 5: Elution diagram of a Sephadex G-50 gel filtration (column 3.2 × 25 cm) of a tryptic digest of a bovine serum albumin sample that had been illuminated in 6 M urea solution. The sample contained 0.20 mol of coupled fluorescein/mol of albumin. Elution patterns: (—) absorbance at 280 nm; (x) absorbance at 502 nm.

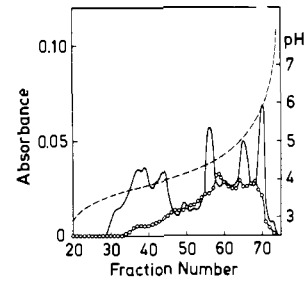

FIGURE 6: SE-Sephadex chromatography elution diagram of fraction II from gel filtration of a tryptic digest of a bovine serum albumin sample that had been illuminated in 6 M urea solution. (- - -) pH. Absorbance was measured at 254 nm (—) and at 502 nm (O) after addition of 100 μl of 25% ammonia to give pH ≥9.

carboxyl and phenolic hydroxyl groups were also found in the adduct and the pK values were also about the same. This indicates that the carboxyl and hydroxyl functions are not involved in the coupling. An important difference, in addition to the change in the absorption maximum, was that the adduct was nonfluorescent at all pH values.

A distinction between the X–F adduct and the tripeptide was that the absorption maximum of the tripeptide at weakly alkaline pH was at 502 as compared to the 499-nm maximum of the adduct. It is known (Laurence, 1952; Andersson *et al.*, 1971b) that the absorption maximum of fluorescein is shifted to the red when transferred from a polar environment to a less polar one and this shift can be taken to indicate that the Leu-Tyr sequence interacts with the fluorescein probably by formation of a hydrophobic complex.

In order to verify the value of the extinction coefficient for coupled fluorescein, assumed to be 8.4×10^4 M^{-1} cm^{-1} at maximal absorption, an experiment was performed in which the fluorescein absorption of the adduct was compared with its content of amino groups. Quantitative measurement of amino groups was performed by reaction of trinitrobenzenesulfonic acid with 100 nmol of X–F adduct as described in the Materials and Methods section. It was found, that if the adduct contains one amino group which is very likely, the extinction coefficient of the coupled fluorescein should be 8.7×10^4 M^{-1} cm^{-1} at 499 nm. It would seem that the coupling of fluorescein has had very little influence on its light absorption properties.

For comparison, an experiment was performed in which 10 ml of a 1% bovine serum albumin solution containing 1 mM fluorescein in 0.02 M sodium phosphate–6 M urea buffer (pH 8.0) was illuminated under the same conditions as in the experiment described above. Excess dye and urea were separated from the protein material by dialysis. The degree of coupling was found to be 0.20 mol of fluorescein/mol of albumin. The protein material was digested with 6 mg of trypsin and separated on a Sephadex G-50 column. The elution diagram is shown in Figure 5. Further separation by ion-exchange chromatography on SE-Sephadex of peptide fraction II obtained from gel filtration showed that the labeling was much less specific than under nondenaturing conditions. The elution curve obtained for the run of fraction II is shown in Figure 6. It is evident that the labeling pattern obtained is to a large degree dependent on the conformation of the protein. In the native conformation of the protein it can be assumed that only the residues available to solvent are able to react. Further specificity may be obtained by preferential labeling of binding sites as seems to be the case with this system.

Discussion

The results obtained in this study show that coupling of dyes to various biopolymers can be accomplished by dye-sensitized photooxidation. In order to get a measurable degree of coupling, however, certain conditions are essential. The concentration of dye must be considerably higher than normally used in dye-sensitized photooxidation experiments and the light intensity has to be fairly high.

Unfortunately, no definite conclusion can be drawn regarding the coupling mechanism. The systems are rather complex and although a large amount of information is available on the photochemistry of fluorescein (Lindqvist, 1960, 1963; Kasche and Lindqvist, 1964, Kasche, 1967), and the other dyes used (Kato *et al.*, 1964; Kellman, 1971), there are still some uncertainties, especially as regards the possible role of singlet oxygen. However, some general outlines can be given on the probable mechanisms of the coupling reaction, which we suppose are similar in many respects to the mechanisms of photosensitized oxidation (for review see Foote, 1968). The first stage of the reaction is the excitation of the dye with light followed by reaction of the excited molecule with oxygen and/or radical formation. Some dye radical species X· or excited form D* of the dye, the peroxy radical HO_2·, or singlet oxygen $^1O_2$ may then react with the biopolymer PH and abstract a hydrogen atom according to reaction 1. The radical P· formed from

$$PH + X\cdot(D^*, HO_2\cdot, {}^1O_2) \longrightarrow P\cdot + XH\ (DH\cdot, H_2O_2, HO_2\cdot) \quad (1)$$

the biopolymer can be expected to be very reactive and a possible further reaction would be the recombination of radical P· with another dye radical X· resulting in the formation of a biopolymer–dye adduct as seen in reaction 2. If

$$P\cdot + X\cdot \longrightarrow PX \quad (2)$$

the biopolymer radical P· reacts with oxygen, peroxy radical, or singlet oxygen, probably no coupling occurs but instead a chemical modification is obtained at the site having an unpaired electron. The actual coupling reaction would thus be a radical–radical reaction. As such it would be expected to show a strong dependency on the light intensity and the dye concentration in good agreement with the results obtained.

The studies on the specificity of the coupling were limited to one system, the bovine serum albumin–fluorescein system. This system was chosen mainly because the binding of fluorescein to bovine serum albumin has been fairly extensively studied (Andersson *et al.*, 1971b). Tryptic digestion of the albumin–fluorescein adduct, followed by separation

of the peptide mixture, showed that one peptide was considerably more labeled than the others. This tripeptide was characterized and from available sequence data on albumin it was suggested that the sequence was Tyr-Leu-Tyr, in whichthe N-terminal trosine contained the coupled fluorescein. This sequence is to be found about 130 amino acid residues from the N-terminal end of the molecule. The environment around this sequence is interesting since it contains a number of hydrophobic amino acid residues and one of the two tryptophan residues of the albumin molecule. The sequence is Lys-Lys-Phe-Trp-Gly-Lys-Tyr-Leu-Tyr-Glu-Ile-Ala-Arg-Arg-His. Several basic amino acids are also fou in this sequence giving an overall picte of a hydrophobic region containing positive charges. This is a type of region which could be expected to bind negatively charged amphipathic substances such as fluorescein or fatty acid anions. indications have earlier been obtained that the two tryptophans in bovine serum albumin are involved in the binding of fatty acid anions (Spector and John, 1968). It is thus clear that the presence of a binding site for fluorescein in this region is very probable and it is likely that the coupling in this case is specific for a binding site. Support is also obtained from the observation that there is a certain degree of hydrophobic interaction with the fluorescein even in the tripeptide.

However, there are two other binding sites for fluorescein present in bovine serum albumin. It has been shown (Andersson *et al.*, 1971b) that one of these binding sites, or possibly both, is situated close to a sequence Leu-Ser-Gln-Lys-Phe-Pro-Lys present about 400 residues from the amino terminal end of the molecule. No labeled peptide corresponding to this sequence or part of it could be isolated, however, despite the fact that we from earlier work have a considerable experience of working with peptides from this sequence. This indicates that very little coupling has taken place in this sequence, since there is no amino acid residue in this sequence that is sensitive to dye-sensitized photooxidation. Our data can be interpreted as indicating that there is a dual specificity in the coupling reaction both with respect to binding site and certain amino acid residues. It should be remembered, however, that the specificity is not absolute. Coupling evidently occurs at sites other than binding sites and it is also possible that coupling can take place with amino acid residues other than those especially sensitive to dye-sensitized photooxidation.

Finally how can the affinity labeling of binding sites be related to the mechanisms of the coupling? There are at least two possible ways to explain this, where one can be seen as a special case of the other. The special case is the possibility that an excited form or a radical species of fluorescein is bound to the binding site and abstracts a hydrogen atom from some residue in the binding site or adjacent to it. This residue then contains an unpaired electron which can react with another radical species of fluorescein to form an adduct. The other possibility, the general case, is that peroxy radicals, singlet oxygen, excited fluorescein molecules, or fluorescein radicals abstract hydrogens more or less randomly from sensitive amino acids on the surface of the protein. Specificity is gained in the second step in which the fluorescein radical preferentially reacts with a radical species of an amino acid residue at the binding site.

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References

Andersson, L.-O., Brandt, J., ad Johansson, S. (1971a), *Arch. Biochem. Biophys. 146,* 428.

Andersson, L.-O., Rehnström, A., and Eaker, D. L. (1971b), *Eur. J. Biochem. 20,* 371.

Brown, J. R., Low, T., Behrns, P., Sepulveda, P., Parker, K., and Blakeney, E. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30,* 1104.

Fields, R. (1971), *Biochem., J. 124,* 581.

Foote, C. S. (1968), *Science 162,* 963.

Gray, W. R. (1967), *Methods Enzymol. 11,* 139.

Hagenfeldt, L. (1966), *Clin. Chim. Acta 13,* 266.

Jori, G., Galiazzo, G., Marzotto, A., and Scoffone, E. (1968a), *Biochim. Biophys. Acta 154,* 1.

Jori, G., Galiazzo, G., Marzotto, A., and Scoffone, E., (1968b), *J. Biol. Chem. 243,* 4272.

Jori, G., Galiazzo, G., and Scoffone, E. (1969), *Biochemistry 8,* 2868.

Kasche, V. (1967), *Photochem. Photobiol. 6,* 643.

Kasche, V., and Lindqvist, L. (1964), *J. Phys. Chem. 68,* 817.

Kato, S., Morita, M., and Koizumi, M. (1964), *Bull. Chem. Soc. Jap. 37,* 117.

Kellman, A. (1971), *Photochem. Photobiol. 14,* 85.

King, T. P., and Spencer, M. (1970), *J. Biol. Chem. 245,* 6134.

Kunitz, M. (1946), *J. Biol. Chem. 164,* 563.

Laurence, D. J. R. (1952), *Biochem. J. 51,* 168.

Lee, J., and Seliger, H. H. (1964), *J. Chem. Phys. 40,* 519.

Lindqvist, L. (1960), *Ark. Kemi 16,* 79.

Lindqvist, L. (1963), *J. Phys. Chem. 67,* 1701.

Parker, C. A. (1953), *Proc. Roy. Soc., Ser. A 220,* 104.

Scoffone, E., Galiazzo, G., and Jori, G. (1970), *Biochem. Biophys. Res. Commun. 38,* 16.

Spector, A. A., and John, K. M. (1968), *Arch. Biochem. Biophys. 127,* 65.

Woods, K. R., and Wang, K.-T. (1967), *Biochim. Biophys. Acta 133,* 369.