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Use of a Solid-Phase Photoaffinity Reagent To Label a Steroid Binding Site: Application to the Δ^5-3-Ketosteroid Isomerase of *Pseudomonas testosteroni*†

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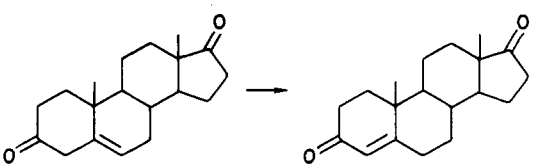
ABSTRACT: In order to extend our analysis of the reactions that occur during the active site directed photoinactivation of Δ^5-3-ketosteroid isomerase sensitized by unsaturated steroid ketone photoaffinity reagents, the site of covalent attachment has been identified. A solid-phase photoaffinity reagent, Δ^6-testosterone–agarose, has been employed for this purpose; this type of reagent, in contrast to solution-phase reagents, facilitated the recovery of a peptide fragment of the isomerase bearing the residue at which covalent attachment had occurred. Amino acid analysis and sequence determination of the peptide provided evidence that the site of attachment was aspartate-38. This result, in combination with the low-resolution crystallographic structure of the enzyme [Westbrook, E. M., Piro, O. E., & Sigler, P. B. (1984) *J. Biol. Chem. 259*, 9096–9103], suggests that aspartate-38 is located in the vicinity of the bottom of the steroid-binding pit. The potential usefulness of solid-phase photoaffinity reagents in the identification of sites of covalent attachment on target proteins such as hormone receptors is discussed.

Some years ago Martyr & Benisek (1973) described a new method of photoaffinity labeling that exploited the photochemical properties of the conjugated unsaturated ketone grouping as a light-activatable reactive entity in photoaffinity reagents. This work was of broad significance to the field of steroid hormone endocrinology since the conjugated enone chromophore is a naturally occurring part of the structure of androgens and progestins. Thus, the natural ligands, themselves, could, potentially, be used as photoaffinity reagents to label their own binding sites (Martyr & Benisek, 1973; Katzenellenbogen & Katzenellenbogen, 1984).

The protein employed to demonstrate the feasibility of using steroid enones as photoaffinity reagents was the Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* (EC 5.3.3.1, steroid Δ-isomerase). This protein affords several advantages as a test system for the development of new methodology in protein chemistry. The enzyme has 125 residues in each of its two identical polypeptides (Benson et al., 1971). Moreover, the enzyme can be purified in good yield from steroid-induced cultures of *P. terstosteroni*. It is stable at neutral pH at temperatures below 50 °C. The sequence of the isomerase polypeptide has been determined by Benson et al. (1971) and partially confirmed by Ogez et al. (1977). A 6-Å resolution crystal structure of the isomerase has been described by Westbrook et al. (1984).

Scheme I



The work of Martyr & Benisek (1975) and of Ogez et al. (1977) has established that the major reaction which occurs during photoaffinity modification of the enzyme with 19-nortestosterone acetate is the photodecarboxylation of aspartate-38, a reaction that does not involve covalent attachment of a steroid moiety to the enzyme. Nevertheless, the decarboxylation inactivates the enzyme; this suggests that the

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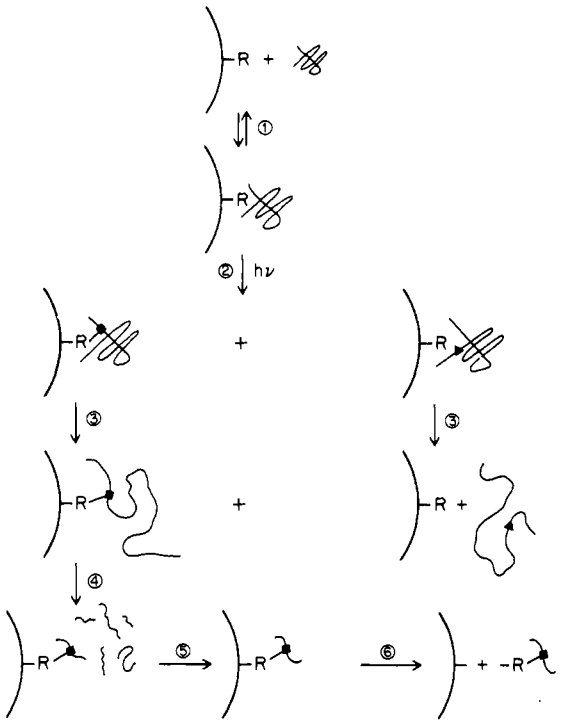


FIGURE 1: Strategy of solid-phase photoaffinity labeling. (1) The target protein is applied to the solid-phase reagent and binds to the reactive moiety R at a specific site. (2) The complex is irradiated. The protein is modified by reactions that covalently couple or do not couple the protein to R. (3) The mixture of products is washed with buffer, competitive inhibitor, and denaturing solution, sequentially. Protein species that have not become covalently attached to R are washed away. Covalently attached species are denatured but remain associated with the solid phase. (4) These species are fragmented to peptides by chemical or enzymatic proteolysis. (5) Peptides that do not contain sites of covalent attachment to R are washed away by a denaturing solvent, whereas peptides that bear such sites remain attached. (6) Covalently attached peptides are recovered by cleavage of a labile bond in the arm which connects R to the solid-phase matrix. (■) indicates a residue that is modified by covalent attachment to R. (▲) indicates a residue that is modified without covalent attachment to R.

carboxylate moiety of Asp-38 is of importance in catalysis or substrate binding (Scheme I).

In addition, these workers detected a minor reaction of an unknown nature that was distinguished from the decarboxylation by the fact that covalent attachment to the protein occurred. The covalent attachment process appeared to be active site specific since competitive inhibitors that do not absorb light above a wavelength of 240 nm inhibited the coupling of radiolabeled testosterone and 19-nortestosterone to the isomerase (Martyr, 1974).

It was of interest to identify the site or sites of covalent attachment since this would provide further information about the structure of the active site, but this has been frustrated by the low efficiency of the coupling reaction. To deal with this problem, Hearne & Benisek (1983) developed a solid-phase photoaffinity reagent, Δ^6-testosterone–agarose, which would, in principle, permit specific retrieval of the small fraction of enzyme molecules that suffered covalent attachment to the steroid during irradiation. Subsequent studies of Hearne & Benisek (1984) localized the site of covalent attachment of the Δ^6-testosterone moieties of the solid-phase reagent to the region between residues 28 and 45 of the isomerase polypeptide.

The strategy embodied in the solid-phase photoaffinity reagent method is displayed in Figure 1. Briefly, the reagent in the form of a 5 × 40 mm minicolumn is complexed with the isomerase in neutral, nondenaturing buffer. The complex is irradiated with light of wavelength longer than 290 nm for a period of time sufficient to inactivate greater than 80% of the isomerase activity. Isomerase molecules that remain unmodified or that are modified without covalent attachment to the steroid moiety can be removed by successive elutions of the solid phase with buffer, competitive inhibitors, and denaturing media. Covalently attached polypeptide can be released at this stage or it can be subjected to proteolysis on the solid phase. After proteolysis, the peptide fragments that do not remain attached can be washed away. The attached fragments can then be recovered as a class by cleavage of a labile arm linking the photoresponsive ligand to the solid phase. The detailed methodology has been described by Hearne & Benisek (1983, 1984).

This report describes new studies designed to pinpoint the site on the enzyme at which covalent attachment of the steroid moiety of Δ^6-testosterone–agarose occurs. Evidence is presented which indicates that Asp-38 is this site.

Materials and Methods

Δ^5-3-Ketosteroid isomerase was purified from progesterone-induced cells of *P. testosteroni* by the procedure of Jarabak et al. (1969) as modified by Benson et al. (1974) and Ogez et al. (1977). The isomerase isozyme having an isoelectric point of 4.75 was used for these studies. The specific activity of the purified enzyme varied between 40 000 and 50 000 units per milligram of protein when assayed as described by Schriefer & Benisek (1984). The enzyme preparation exhibited only one discernible Coomassie Blue staining band when analyzed by SDS[1]–polyacrylamide gel electrophoresis in the presence of 8 M urea.

Δ^6-Testosterone hemisuccinate was purchased from Steraloids. Sodium cholate was a product of Sigma. The isomerase substrate androst-5-ene-3,17-dione was synthesized from 3β-hydroxyandrost-5-en-17-one (Steraloids) by the method of Djerrassi et al. (1956). *O*-(Carboxymethyl)agarose was obtained from Bio-Rad as CM-Bio-Gel A. Ethylenediamine was a product of Mallinckrodt and was used without further purification. Δ^6-Testosterone succinylethylenediamine–*O*-(carboxymethyl)agarose (Δ^6-testosteorone–agarose) was synthesized as described previously (Hearne & Benisek, 1983). Triethylamine was sequenal grade of Pierce and was distilled from dansyl chloride before use. Guanidinium chloride, grade 1, was from Sigma. TLCK-treated α-chymotrypsin was purchased from Sigma. Constant-boiling HCl was prepared from 6 N HCl by double distillation. Phenol was obtained as crystals from Mallinckrodt. Sephadex G-15 was from Pharmacia.

Photoaffinity Labeling. In a typical experiment, a total of 5.9 mg of isomerase was applied to 40 minicolumns (5 × 40 mm) of Δ^6-testosterone–agarose in 0.3 M potassium phosphate buffer, pH 7.0. The columns were purged with nitrogen-saturated 0.3 M phosphate buffer to remove oxygen. The columns were irradiated with a Pyrex-filtered medium pressure mercury discharge lamp as described for solution-phase labeling by Benisek (1977). Following the irradiation, each column was eluted sequentially with 2 mL of 0.3 M potassium phosphate, pH 7.0, two 3-mL portions of 0.5 mM sodium cholate in 0.3 M potassium phosphate, pH 7.0, 4 mL of water, 4 mL of 6 M guanidinium chloride, and finally 10 mL of

[1] Abbreviations: SDS, sodium dodecyl sulfate; TLCK, N^{α}-*p*-tosyllysine chloromethyl ketone; TEA, triethylamine; HPLC, high-performance liquid chromatography.

water. Twelve additional columns were prepared similarly, but no isomerase was applied, since they were to serve as enzymeless controls. The controls were irradiated at the same time as the experimental columns and were subjected to the same elution protocol.

Chymotryptic Digestion of Covalently Bound Isomerase. The agarose from the experimental columns was pooled into groups derived from five columns. Each pool was digested by treatment with a solution composed of 3.3 mL of buffer (0.1 M sodium phosphate, 0.1 mM $CaCl_2$, pH 7.6), 2 μL of toluene, and 25 μL of 0.25 mg/mL chymotrypsin in 1 mM HCl. The suspensions were incubated for 24 h at 30 °C with stirring.

Recovery of Covalently Bound Peptides. The eight digestion mixtures were combined into a single pool that was poured into a 2-cm diameter column. This was washed with a total of 40 mL of 6 M guanidinium chloride and then with 300 mL of water.

The column was unpacked and the attached peptide material cleaved from the agarose phase by incubation with 25 mL of 1% TEA–HOAc, pH 10.5, for 3 h at 30 °C with stirring. Separate experiments with unirradiated Δ^6-testosterone–agarose showed that this treatment completely hydrolyzed the succinyl ester bond of the reagent arm. The suspension was centrifuged briefly and the supernatant solution decanted and retained. The agarose was washed twice with a total of 15 mL of the pH 10.5 TEA–HOAc buffer, and the washes were combined with the first supernatant solution. The combined TEA eluates were lyophilized. The residue was redissolved in 300 μL of 0.1 M ammonium bicarbonate and the resulting solution applied to a 1 × 57.5 cm column of Sephadex G-15 equilibrated with the same solution. The column was eluted with the same solution of ammonium bicarbonate at a flow rate of 1.8 mL/h while 0.3-mL fractions were collected. The absorbance of the fractions at 210 nm was measured. The enzymeless control was processed similarly; however, the solvent volumes were scaled to the smaller number of minicolumns utilized. The peaks that eluted from these columns were pooled and thoroughly lyophilized in order to remove excess ammonium bicarbonate prior to further study.

Amino Acid Analysis. Each sample for amino acid analysis was prepared by lyophilization to a dry residue in a Pyrex ignition tube. Constant-boiling HCl and a small crystal of phenol were added to the tube, which was then vacuum degassed and sealed in vacuo. Hydrolysis was allowed to proceed at 110 °C for 24 h. The hydrolysate was evaporated to dryness over NaOH pellets in an evacuated desiccator. The residue was taken up in pH 2.2 sample buffer and the resulting solution analyzed for amino acids with either a Durrum D-500 or a Beckman 6300 amino acid analyzer.

Peptide Sequencing. Peak A from the gel chromatography step was sequenced with a Beckman Model 890M sequencer, which was under the control of the 0.1 M Quadrol program 050783. Polybrene (2 mg) was added to the cup in order to reduce extractive losses. The peptide sample (see Results) was applied as a solution in 80% formic acid. Twenty-eight cycles of Edman degradation were performed. PTH-amino acids were identified by two different HPLC methods. The primary identifications were made according to the method of Bhown et al. (1978) using a 4.6 × 250 mm, 5-μm particle size, C-18 reverse-phase column from Altex. Chromatography and quantitation were performed by using a system composed of two Waters 6000-A pumps, a Waters Wisp autosampler, a Waters Model 440 detector operated at 254 nm, and a Waters data module. Yields of PTH-amino acids were corrected for the background level determined for the preceding residue and

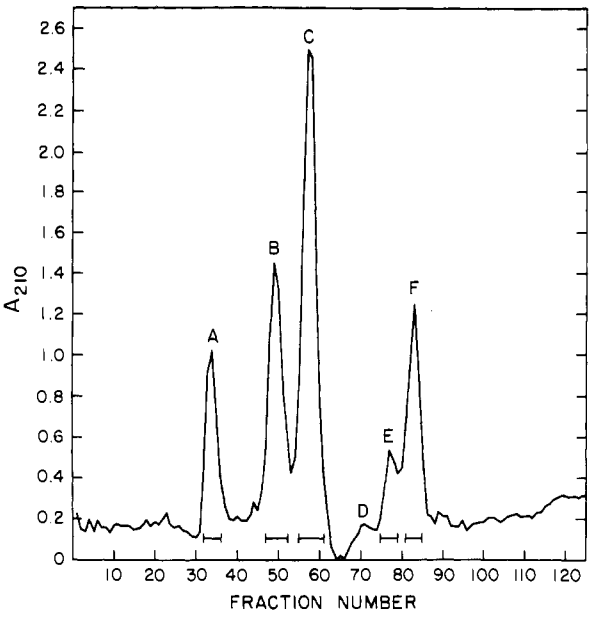


FIGURE 2: Chromatography of covalently attached chymotryptic peptides from experimental columns on Sephadex G-15. The conditions of chromatography are described under Materials and Methods. Fractions were pooled as indicated by the horizontal bars. Amino acid analysis of the pooled fractions indicated that only peak A contained significant quantities of peptide material.

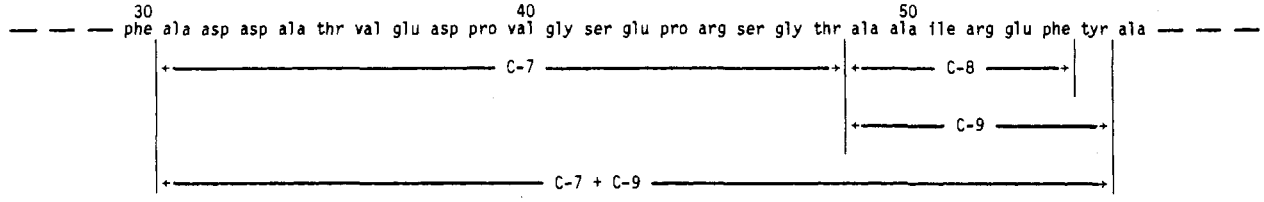
were normalized to the measured amount of PTH-Nle used as the internal standard. It was not possible to reliably quantitate PTH-Gly, Ser, and Thr due to their typically poor recoveries and distribution among multiple peaks. The identifications based on these analyses were qualitatively confirmed by a separate HPLC analysis using the method of Hunkapiller & Hood (1983). Chromatography was performed on a 4.5 × 250 mm cyanoethyl reverse-phase column (IBM Analytical Instruments). The chromatograph consisted of a Perkin-Elmer Series 400 solvent delivery system, a Waters Wisp autosampler, and a Kratos 783 variable wavelength detector set to monitor at 269 nm.

RESULTS

Photoinactivation by Δ^6-Testosterone–Agarose. In previous studies Hearne & Benisek (1984) observed that photoinactivation of the isomerase by Δ^6-testosterone–agarose was accompanied by the formation of three elution classes of isomerase protein: a class eluted with sodium cholate, a class subsequently eluted with denaturants such as SDS, and a class that was subsequently eluted with pH 10.5 buffer treatment. In the present preparative-scale experiments, 10.7% of the total applied isomerase activity failed to bind to the solid-phase reagent and was washed out by the nitrogen-saturated phosphate buffer prior to irradiation. After irradiation, an additional 14.9% of the applied activity was eluted by the phosphate buffer wash and 0.8% more by the succeeding cholate wash. Active enzyme would not be expected in the guanidinium chloride wash since the isomerase is denatured in this solvent.

Isolation of Covalently Attached Chymotryptic Fragments. In smaller scale experiments Hearne & Benisek (1984) had found that ca. 20% of the applied protein was released by the pH 10.5 hydrolysis procedure. After chymotryptic digestion as described under Materials and Methods and removal of the soluble peptides, the material released by the pH 10.5 hydrolysis was separated from low molecular weight components that interfered with Edman sequence analysis by Sephadex G-15 chromatography. The chromatographic profile for the experimental sample is shown in Figure 2. Six peaks, A–F,

Scheme II: Amino Acid Sequence of Steroid Isomerase in the Vicinity of Chymotryptic Peptides C-7, C-8, and C-9



Scheme III: Amino Acid Sequence of Peak A Peptide

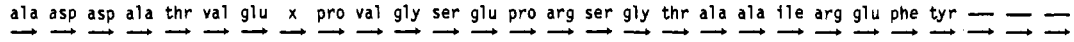


Table I: Amino Acid Composition of Peaks A

amino acid	amount (nmol) peak A (exptl)	peak A (control)	peak A (net)	mole ratio (Pro = 2) peak A (net)	C-7	C-7 + C-8	C-7 + C-9
Asp	6.3	0.7	5.6	2.0	3	3	3
Thr	5.5	0.2	5.3	1.9	2	2	2
Ser	5.2	0.7	4.5	1.6	2	2	2
Glu	7.7	1.0	6.7	2.4	2	3	3
Pro	5.8	0.3	5.5	2.0	2	2	2
Gly	7.3	2.5	4.8	1.7	2	2	2
Ala	9.1	0.5	8.6	3.1	2	4	4
Val	5.5	0.0	5.5	2.0	2	2	2
Met	0.0	0.0	0.0	0.0	0	0	0
Ile	1.5	0.1	1.4	0.5	0	1	1
Leu	0.6	0.3	0.3	0.1	0	0	0
Tyr	0.9	0.0	0.9	0.3	0	0	1
Phe	1.6	0.0	1.6	0.6	0	1	1
His	0.0	0.0	0.0	0.0	0	0	0
Lys	0.3	0.3	0.0	0.0	0	0	0
Arg	4.6	0.0	4.6	1.7	1	2	2

were observed. A similar pattern was observed in the control chromatogram except that peaks A, E, and F were much smaller. Amino acid analysis was performed on acid hydrolysates of the pooled peak fractions in order to ascertain which of the peaks contained peptides and to begin the characterization of any peptides present. The only peaks that contained significant amounts of peptide-like material were peaks A from the experimental and control samples. Peaks A eluted at the excluded volume of the column. The amino acid compositions of the experimental and control peaks A were qualitatively and quantitatively quite different, as described below.

Amino Acid Composition of Peak A. The amino acid compositions of peaks A from the experimental and control samples are given in Table I, columns 2 and 3, and the net composition, obtained by subtracting the control from the experimental values, is given in column 4. The corresponding mole ratios relative to proline are shown in column 5. Comparison of the data of column 5 with the theoretical compositions of chymotryptic peptide C7 and the double chymotryptic peptides C-7 + C-8 and C-7 + C-9 indicated that, with the exception of aspartic acid, peak A was an approximately equimolar mixture of C-7 and C-7 + C-9. The sequence of isomerase in the vicinity of these fragments is shown in Scheme II. However, only two residues of aspartic acid were observed rather than the three residues expected for unmodified C-7 or for C-7 + C-9. The total amount of peptide recovered from the agarose phase was 13.5 nmol.

Amino Acid Sequence of Peak A. Without further purification of the material comprising peak A, 4600 pmol of the putative mixture was subjected to sequence analysis by automated Edman degradation, as described under Materials and Methods. The HPLC system of Bhown et al. (1978) was able to identify a PTH-amino acid at each cycle of the degradation, except for cycle 8, for which no PTH-amino acid could be detected above the background. The sequence obtained is

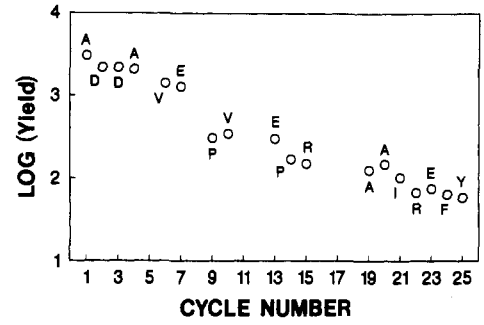


FIGURE 3: Edman degradation of peak A. PTH-amino acids were identified and quantitated as described under Materials and Methods. The repetitive yield of the degradation through cycle 7 was 87% while that for residues 9–25 was 91%. No PTH-amino acid was detected at cycle 8. The degradation proceeded through the C-terminal tyrosine since no PTH-amino acids were detected at cycles 26–28.

shown in Scheme III. The absence of a PTH-amino acid at cycle 8 was also observed by using the HPLC system of Hunkapillar & Hood (1983). Furthermore, an abrupt decrease in the yield of all PTH-amino acids beyond cycle 8 was noted. A plot of the yields of many of the PTH-amino acids as a function of cycle number is shown in Figure 3. Material obtained from an independent, similar photoaffinity labeling reaction gave the same sequencing results. In the latter study, the PTH-amino acids of cycles 7, 8, and 9 were additionally analyzed by the peptide chromatographic system of Mahoney & Hermodsen (1980), which utilizes a C-18 reverse-phase column and a propanol gradient. Even this system, with its greater ability to elute hydrophobic solutes, failed to detect any PTH-amino acid at cycle 8.

Discussion

Site of Covalent Attachment. Previous studies by Hearne & Benisek (1983, 1984) have established that the photoinactivation of steroid isomerase sensitized by Δ^6-testosterone–agarose is an active site event since the competitive inhibitor sodium cholate inhibits inactivation. Inactivation was found to consist of two processes, the quantitatively lesser of the two involving covalent attachment of the protein to the solid-phase reagent. Hearne & Benisek (1984) found that the covalent attachment reaction was suppressed by cholate; this indicates that this component of the inactivation was active site selective. Similar results had been obtained earlier in solution-phase studies employing 19-nortestosterone acetate and testosterone as the photoaffinity reagents (Martyr & Benisek, 1973, 1975; Martyr, 1974).

The identity of the chymotryptic fragment that remained associated with the agarose phase was first suggested to be the double chymotryptic peptide C-7 + C-9 mixed with an equimolar quantity of C-7. This conclusion is based on the amino acid composition shown in Table I, column 5, which resembles that expected for C-7 but exhibits excess quantities

of just those amino acids that are present in C-9. The excess quantities are in the correct proportion to be completely consistent with this source peptide. Noteworthy, however, is the shortfall of one residue of aspartic acid; this suggests that aspartic acid is the site of covalent attachment.

These tentative conclusions are completely confirmed by the results of the Edman degradation, Scheme III and Figure 3, which yielded a clean, single sequence of the peptide material in peak A. This sequence agreed with that expected for C-7 + C-9 with the exception of the blank at cycle 8 and the 4-fold drop in yield of PTH-amino acids between cycles 7 and 9. Comparison of the sequence of Scheme III with that of the portion of the native protein shown in Scheme II identifies Asp-38 as the site of modification, presumably by covalent attachment to the steroid moiety of the solid-phase reagent. This conclusion is consistent with the amino acid composition results, which showed a loss of one residue of aspartic acid. It should be noted that sequence analysis of C-7 from native isomerase (Ogez et al., 1977) clearly identified residue 38 as aspartic acid. In addition, cycles 2 and 3 of the Edman degradation of peak A resulted in acceptable yields of PTH-aspartic acid. The cause of our failure to detect the PTH derivative of the modified Asp-38 or for the precipitous drop in the yield of PTH derivatives of subsequent residues is not apparent at this time. We conclude from these results that photomodification of Asp-38, presumably by a reaction involving covalent attachment of this residue to the solid-phase reagent, was the attachment process detected by our earlier studies.

Location of Asp-38 in the Substrate Binding Site. Westbrook et al. (1984) have obtained a 6-Å resolution crystal structure of the steroid isomerase of *P. testosteroni* and have located a steroid binding site on each monomer of the enzyme dimer. Difference electron density maps of 4-(mercuriacetoxy)-17β-estradiol plus isomerase minus isomerase place the steroid binding site near the subunit interface. The site can be described as a pit, with the mercury atom of the bound steroid positioned close to a projection of electron density extending from the other subunit. Since the mercury atom is on the A ring, Westbrook et al. pointed out that if this was a productive binding mode, the catalytic groups of the protein would be found in the vicinity of the mercury atom and might be contributed by residues from both subunits. Figure 6 of Westbrook et al. (1984) indicates that the region of the steroid binding pit where both subunits could provide groups to the site is located on the surface of the protein globule.

Considerable evidence now exists that Asp-38 is an important residue in the catalytic process. Ogez et al. (1977) found that the major reaction which occurs during the photoinactivation of the isomerase sensitized by 19-nortestosterone acetate is the decarboxylation of Asp-38. Benisek et al. (1980) reported that amidation of the enzyme with cystamine and a carbodiimide occurs mainly at Asp-38; this results in an inactive enzyme. Kayser et al. (1983) have demonstrated that 17β-steroid oxiranes alkylate Asp-38 in a site-specific manner and that 3β-steroid oxiranes alkylate a carboxylic residue in the vicinity of Asp-38, possibly Asp-38, itself; both reactions result in inactive enzyme derivatives (Bevins et al., 1984). In view of these observations, the location of Asp-38 in the steroid binding pit is of interest.

The amino acid analysis and sequencing results of the present study clearly demonstrate that Asp-38 is the site of modification by the solid-phase photoaffinity reagent Δ^6-testosterone–agarose. Consideration of the stereochemical consequences of the macromolecular agarose polymer attached to the 17β position on the D ring of a steroid makes it seem unlikely that the steroid moiety of the solid-phase reagent could bind to the isomerase with the A ring on the surface and the D ring at the base of the pit. A much more reasonable binding mode would place the A ring at the base of the pit and the D ring at the protein surface, an orientation opposite to that observed by Westbrook et al. for bound 4-(mercuriacetoxy)-17β-estradiol. The A and B rings of the Δ^6-testosterone moiety contain the photoreactive group of the reagent. Thus, we expect that Asp-38 is positioned close to this region of the bound steroid moiety of the agarose reagent. These considerations lead us to the conclusion that Asp-38 is located at or near the base of the steroid binding pit; this raises the possibility that the catalytic groups will be found in this region, also.

It is now well documented that the site specificity of labeling of steroid binding sites by steroid enones is often quite high but that the efficiency of covalent attachment is usually less than 20% (Katzenellenbogen & Katzenellenbogen, 1984). This low efficiency is not a problem for those studies that aim to identify steroid receptors in crude cell extracts since the receptors bind with such high affinity and the photoreagents can be obtained as tritium-labeled forms of high specific activity. However, for those studies that are directed to the identification of specific peptide regions or specific amino acid residues in hormone binding sites, the low efficiency of covalent attachment is a major impediment to progress. It is to the latter type of study that the application of solid-phase photoaffinity reagents may have a most immediate application. Importantly, receptor preparations need not be highly purified in order for the solid-phase photoaffinity labeling strategy to be fruitful.

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Picosecond Kinetics of the Initial Photochemical Electron-Transfer Reaction in Bacterial Photosynthetic Reaction Centers†

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ABSTRACT: The absorption changes that occur in reaction centers of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* during the initial photochemical electron-transfer reaction have been examined. Measurements were made between 740 and 1300 nm at 295 and 80 K by using a pulse–probe technique with 610-nm, 0.8-ps flashes. An excited singlet state of the bacteriochlorophyll dimer P* was found to give rise to stimulated emission with a spectrum similar to that determined previously for fluorescence from reaction centers. The stimulated emission was used to follow the decay of P*; its lifetime was 4.1 ± 0.2 ps at 295 K and 2.2 ± 0.1 ps at 80 K. Within the experimental uncertainty, the absorption changes associated with the formation of a bacteriopheophytin anion, Bph^-, develop in concert with the decay of P* at both temperatures, as does the absorption increase near 1250 nm due to the formation of the cation of P, P^+. No evidence was found for the formation of a bacteriochlorophyll anion, $Bchl^-$, prior to the formation of Bph^-. This is surprising, because in the crystal structure of the *Rhodopseudomonas viridis* reaction center [Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1984) *J. Mol. Biol. 180*, 385–398] a Bchl is located approximately in between P and the Bph. It is possible that $Bchl^-$ (or $Bchl^+$) is formed but, due to kinetic or thermodynamic constraints, is never present at a sufficient concentration for us to observe. Alternatively, a virtual charge-transfer state, such as P^+Bchl^-Bph or $PBchl^+Bph^-$, could serve to lower the energy barrier for direct electron transfer between P* and the Bph.

Reaction centers from *Rhodopseudomonas sphaeroides* contain three polypeptide chains with a total M_r of about 80000, four bacteriochlorophylls (Bchl), two bacteriopheophytins (Bph), one or two ubiquinone molecules (Q_A and Q_B) depending on the preparation, and a non-heme iron atom. Recently, the three-dimensional structure of the reaction center from a related bacterium, *Rhodopseudomonas viridis*, has been solved to 3-Å resolution (Figure 1) (Deisenhofer et al., 1984). Two of the Bchls are associated to form a special pair (P). The other two Bchls ($Bchl_L$ and $Bchl_M$) are placed symmetrically on either side of P, followed by the two Bph molecules (Bph_L and Bph_M) and the quinones. When the reaction center is excited with light, an electron is transferred from P to Bph_L, forming the radical-pair state P^+Bph^- in about 5 ps (Zinth et al., 1985; Shuvalov et al., 1978; Holten et al., 1980; Dutton et al., 1975; Shuvalov & Klevanik, 1983; Parson & Ke, 1982). An electron then moves from Bph^- to Q_A in about 200 ps (Parson & Ke, 1982).

The crystal structure (Figure 1) suggests that the initial charge separation proceeds by transfer of an electron first from P to $Bchl_L$ and then from there to Bph_L. In agreement with this hypothesis, pulse–probe measurements using 30-ps pulses have suggested that the transient state P^+Bchl^- may be created prior to P^+Bph^- (Shuvalov et al., 1978; Shuvalov & Klevanik, 1983). However, the evidence on this point has been ambiguous (Kirmaier et al., 1985a; Borisov et al., 1983), and the role of $Bchl_L$ in the electron-transfer sequence is not yet clear.

In this work we use picosecond pulse–probe techniques to investigate the kinetics and spectra of absorbance changes between 740 and 1300 nm at 295 and 80 K. We follow the decay of an excited singlet state of P (P*) by monitoring stimulated emission and find that this decay occurs with the same time constant as the formation of the state P^+Bph^-. We find no evidence for the formation of the state P^+Bchl^- between P* and P^+Bph^-. Preliminary data have been presented previously (Parson et al., 1985a).

Materials and Methods

Reaction centers were prepared from *Rps. sphaeroides* as described previously (Schenck et al., 1982) and were suspended in 0.1% lauryldimethylamine oxide (LDAO) and 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, for measurements at 295 K. For some low-temperature measurements, 56% (v/v) glycerol was added. For most low-temperature measurements, reaction centers were placed in polyvinyl alcohol (PVA) films essentially as described (Schenck et al., 1981a). For measurements involving reduction

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