On the Photoaffinity Labeling of Rabbit Muscle Phosphofructokinase with $O^{2'}$-(Ethyl-2-diazomalonyl)adenosine 3′:5′-Cyclic Monophosphate†

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ABSTRACT: Incomplete labeling has been a characteristic drawback of work with photoaffinity reagents. In this paper two methods for increasing the extent of photochemical labeling of rabbit muscle phosphofructokinase with $O^{2'}$-(ethyl-2-diazomalonyl)adenosine 3′:5′-cyclic monophosphate by irradiation at 253.7 nm are presented. Both involve continuous replacement of photolyzed material with fresh diazo compound and both succeed in giving considerable enrichment of labeling. The effects of prolonged irradiation at 253.7 nm on several properties of phosphofructokinase are examined. Initial studies are made comparing the kinetic properties of native and covalently modified enzyme. Modified enzyme is still found to be subject to ATP inhibition, but is only weakly reactivated by adenosine 3′:5′-cyclic monophosphate.

In a preliminary communication (Brunswick and Cooperman, 1971) we described the synthesis of several ethyl-2-diazomalonyl derivatives of adenosine 3′:5′-cyclic monophosphate (cAMP)[1] which could serve as photoaffinity labels, and showed that one of them, $O^{2'}$-(ethyl-2-diazomalonyl)adenosine 3′:5′ cyclic monophosphate (I), was covalently incorporated into rabbit muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) on irradiation with ultraviolet light. We were unable to achieve quantitative labeling even with saturating concentrations of I. This failure is common to studies with photoaffinity labels. Although there are clear advantages in the use of photoaffinity labels in the study of receptor site interactions, very low levels of covalent incorporation could make site isolation studies difficult, while quantitative labeling could often be necessary for studies on the effect of covalent binding on enzymatic properties. There was therefore a need for general methods to increase the amount of labeling obtained in photoaffinity studies. In this paper we present two methods which we have used to greatly increase the photolytic incorporation of I into phosphofructokinase which should be of general utility in studies with photoaffinity reagents. In addition, we present some initial studies on the kinetic properties of covalently modified enzyme.

Experimental Section

Rabbit muscle phosphofructokinase was obtained from Sigma as an ammonium sulfate suspension. Prior to use it was centrifuged, washed with 60% saturated ammonium sulfate, and treated with acid-washed activated charcoal–Celite (1:1) to remove bound ATP, giving an $A_{280}:A_{260}$ ratio of 1.6 ± 0.1. Tritiated I was prepared as described elsewhere (Brunswick and Cooperman, 1971, 1973). All other regents were obtained from common commercial sources.

All experiments were carried out at 0–4° unless otherwise indicated.

Simple photolysis experiments were performed as previously described (Brunswick and Cooperman, 1971) with the following changes. Photolyses at 253.7 nm were done either in a Rayonet RPR 100 apparatus or with a UVS 11 Mineralight lamp (Ultraviolet Products, Inc.). After irradiation, protein was precipitated by addition of an equal volume of 20% trichloroacetic acid and washed twice with 10% trichloroacetic acid. The precipitate was redissolved in 0.1 M NaOH, enzyme concentration was determined from $A_{280\ nm}$ (in basic solution, $A_{280\ nm}^{0.1\%}$ is 1.02; at this wavelength, the contribution from covalently bound, photolyzed I is negligible), and the sample was counted for radioactivity as described previously (Brunswick and Cooperman, 1971).

Dialysis–photolysis experiments were performed in an apparatus similar to that described by Colowick and Womack (1969) for rapid measurement of ligand binding, except that a circular quartz window (1.5 mm diameter) was cut into the upper, enzyme-containing half of the cell, to allow photolysis of the enzyme–I complex. The cell itself (Bel-Art Products) is made of a plastic which is opaque to light at 253.7 nm. Photolysis was accomplished by means of a UVS 11 light placed horizontally flush with the upper half of the cell and in line with the window. The bottom half of the cell was connected to a reservoir which was continually recirculated by means of a peristaltic pump. The volume of the upper half of the cell was 1.5 ml, while the total volume of the reservoir was 15 ml. Dialysis membrane was prepared as follows: dialysis tubing was soaked 1 hr in 10 mM $NaHCO_3$, boiled for 0.5 hr in fresh 10 mM $NaHCO_3$, soaked in 64% $ZnCl_2$ for 8 min, washed with 0.01 M HCl, and washed with 1 mM EDTA. Membranes thus treated had a $t_{1/2}$ for passage of cAMP of 1 hr, while 7% of bovine serum albumin escaped over a period of 2 days. Treated membranes were stored in 1 mM EDTA containing 0.01% NaN_3. A typical experiment was done as follows. A solution (1.5 ml) of enzyme in buffer A [25 mM glycerophosphate–25 mM glycylglycine–1 mM EDTA–1 mM dithiothreitol–0.2 M ammonium sulfate (pH 7.0)] containing I was placed in the top half of the cell, and 15 ml of buffer A containing I (at the same concentration as above) was placed in the reservoir. The ultraviolet (uv) light and peristaltic pump were turned on, and dialysis–photolysis was allowed to proceed for several hours (Table II). At the end of this time, an aliquot was taken

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[1] Abbreviation used is: cAMP, adenosine 3′:5′-cyclic monophosphate.

TABLE I: Covalent Binding of I to Phosphofructokinase on Irradiation at 253.7 nm.[a]

No.	[I] (μM)	[Pre-photolyzed I][b] (μM)	[Enzyme][c] (μM)	% cAMP Sites Labeled
1	46		10.0	18.0 ± 1.4
2	46	19	10.0	8.5 ± 1.0
3	46	46	10.0	5.9 ± 1.0
4	46	178	10.0	2.9 ± 1.0
5	2.5		100	43 ± 2[d]

[a] In buffer A. [b] Nonradioactive. [c] Based on an equivalent weight of 90,000 for cAMP binding (Kemp and Krebs, 1967) and $A^{0.1\%}_{280\ nm} = 1.02$ at 279 nm (Parmeggiani *et al.*, 1966); experiments 1–4, phosphofructokinase activity, 230 units/mg; experiment 5, phosphofructokinase activity, 170 units/mg. [d] Per cent of radioactivity bound to enzyme.

TABLE II: Covalent Binding of I to Phosphofructokinase by the Dialysis–Photolysis Method.[a]

No. of Cycles	Duration of Cycle[b,c] (hr)	% cAMP Sites Labeled
1	16	42 ± 3
2	16	59 ± 4
3	6	76 ± 5
5	6	67 ± 5
7	18	74 ± 5
d		22 ± 2

[a] The enzyme concentration in the upper half of the cell was 60 μM, calculated as in Table I. The concentration of I was 500 μM. [b] The durations of cycles 4 and 6 were 16 and 18 hr, respectively. [c] The half-life for photolysis of I (500 μM) in the dialysis chamber was approximately 2 hr. [d] Refers to simple photolysis of 10 μM enzyme and 300 μM I in a quartz cell.

and the counts incorporated per weight of protein were determined as described above. The reservoir was emptied and refilled with fresh buffer A containing I. The pump was turned on and dialysis was allowed to proceed for 3 hr to permit replacement of photolyzed I in the upper half of the cell by fresh I. The uv light was then turned on, and a new cycle of dialysis–photolysis begun. This procedure was repeated until a maximum level of incorporation was achieved.

A typical column photolysis experiment was performed as follows. Enzyme was dissolved in buffer B [0.1 M Tris-phosphate–1 mM dithiothreitol–1 mM EDTA (pH 8.0)] and ammonium sulfate removed on a Sephadex G-25 column which had been equilibrated with buffer B. To 1.9 ml of enzyme solution (1 mg/ml) was added 1.2 ml of DEAE-Sephadex A-50 which had been swollen in buffer B, and the mixture was gently stirred and packed into three quartz columns (26 mm × 5 mm) such that each had a final volume of 0.3 ml. Gel adhering to the inside of the column above the level of the gel surface was removed with a damp Kimwipe. The columns were taped parallel to each other in the vertical direction onto the filter of a UVS 11 Mineralight and the filter was mounted onto the horizontally aligned uv lamp such that the entire length of gel fell within the lamp diameter (16 mm). Aluminum foil was placed behind the gel-containing part of the column to act as a reflector. Foil was also wrapped around the columns above the level of the gel to prevent photolysis of the compound before it entered the gel, and below the level of the gel to prevent photolysis of the compound after it emerged from the gel, so that the extent of photolysis occurring on the column could be monitored by measuring the uv spectrum of the eluent. I in buffer B was passed through the columns at a rate of 0.33–0.50 ml/min. Under these conditions enzyme adheres tightly to the column while I is not retained. The uv light was turned on after 1 ml of buffer had passed through each column. After photolysis, to remove noncovalently bound nucleotide, each column was washed first with 8 ml of buffer B containing 0.2 mM cAMP and then with 3 ml of buffer B. Enzyme was eluted with 2 ml of 1 M of Tris-phosphate buffer (pH 8.0), and samples were tested for enzyme concentration (by $A_{280\ nm}$ measurement) and radioactivity. As a check, the per cent incorporation was also determined *via* the trichloroacetic acid procedure described above. The two methods gave essentially the same results.

Measurements of phosphofructokinase activity at pH 7.1 and 25° were made using a modification of the procedure of Hofer and Pette (1968). The assay mixture contained 50 mM glycylglycine, 0.08 mM fructose 6-phosphate, 0.16 mM NADH, Mg^{2+}, ATP, and cAMP (as indicated under Results), 0.1 unit/ml of fructose-1,6-diphosphate aldolase (EC 4.1.2.6), 1.2 unit/ml of triosephosphate isomerase (EC 5.3.1.1), 0.23 unit/ml of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), and 5 mM ammonium sulfate (from the auxiliary enzymes). Measurements of activity at pH 8 were made using the assay described by Ling *et al.* (1966).

Results

Covalent Binding to Phosphofructokinase without Replacement of Photolyzed I. In simple photolysis experiments with I and phosphofructokinase we have previously shown (Brunswick and Cooperman, 1971) that incorporation (a) reaches a saturating value of about 35% of the cAMP binding sites present, (b) depends on carbene formation, and (c) is irreversible. Control experiments demonstrated the validity of our counting procedure and the nondestruction of the cAMP site by a 1-min irradiation (see also Table V). We also showed that incorporation was specific for the cAMP site, using as evidence the decrease in incorporation in the presence of cAMP.[2] These experiments are extended in Table I. Experiments 1–4 show the apparent competitive effect of prephotolyzed I on the incorporation of I, from which it appears that the prephotolyzed material binds noncovalently somewhat more tightly than I. Under the conditions of experiment 5, about 90% of the total I present is noncovalently bound to enzyme, so that the per cent of label incorporated provides an estimate of the probability that, on photolysis, bound I will react with enzyme as opposed to solvent.

Covalent Binding to Phosphofructokinase with Replacement of Photolyzed I. DIALYSIS–PHOTOLYSIS LABELING EXPERIMENT.

[2] It has since been pointed out to us (S. J. Singer, private communication) that incorporation would also be decreased if excess cAMP were to act as a uv filter. If this were so, then, in the presence of excess cAMP, incorporation should increase with time until the original level was obtained. In fact, we have found the low level of incorporation in the presence of cAMP to be unaffected by irradiation for times much in excess of our standard irradiation time of 1 min, so that our demonstration of specificity remains valid.

TABLE III: Covalent Binding of I to Phosphofructokinase on Complete Photolysis in a Quartz Cell Following Partial Photolysis in the Dialysis Cell.[a]

Irradiation Time in Dialysis Cell (min)	% cAMP[b] Sites Labeled	% cAMP Sites[b] Labeled on Complete Photolysis
0	1.7 ± 1.0	24.6 ± 2.0
5	5.5 ± 1.0	22.7 ± 1.8
15	10.3 ± 1.0	18.3 ± 1.5
30	13.4 ± 1.1	17.2 ± 1.4
110	16.2 ± 1.3	18.2 ± 1.5

[a] The enzyme concentration in the upper half of dialysis cell was 9.6 μM, calculated as in Table I. The concentration of I was 51.6 μM; the lower half of the cell was empty. At this concentration of I, $t_{1/2}$ for photolysis was 15 min. [b] After photolysis for the indicated time in the dialysis apparatus the per cent incorporation was determined directly for one aliquot, while a second aliquot was transferred to a quartz cell cuvet and photolyzed to completeness (1 min) in the Rayonet reactor and the per cent incorporation was then determined.

TABLE IV: Covalent Binding of I to Phosphofructokinase by the Column Photolysis Method.[a]

Vol of Buffer B[b] Passed through Column (ml)	Time[c] (min)	% Enzymatic[d] Act. Remaining	% cAMP Sites Labeled
2.5	6		25.5 ± 2.0
6	15		34.0 ± 2.8
15	30	73 ± 3	52.8 ± 4.3
30	60	80 ± 4	63.8 ± 5.2
75	190	34.5 ± 1.5	66.2 ± 5.3
30[e]	60		3.4 ± 1.0
f	10		16.9 ± 1.4

[a] The enzyme concentration on the column was 17 μM, calculated as in Table I. [b] Containing 48 μM I. [c] $t_{1/2}$ for photolysis of I on the column was 1.5 ± 0.5 min. [d] pH 8 assay. [e] In the absence of photolysis. [f] Incorporation obtained on photolysis without replacement of buffer.

The results of a dialysis–photolysis experiment with I are shown in Table II, from which it can be seen that the extent of labeling rose to a maximum value of 70–75% after three cycles. Sixteen hours of dialysis–photolysis were found to give maximum labeling for each cycle. This represents a considerable enrichment over the labeling obtained at near-saturing concentrations of I on photolysis without dialysis in a quartz cell (22%). It is interesting to note that when photolysis is done with a low intensity of incident light over a relatively long period of time, such as in the dialysis apparatus, the level of incorporation is lower than that obtained under conditions of more rapid photolysis (Table III). This phenomenon is not presently understood, but may be due to a slow, dark process following photolysis.

DEAE-SEPHADEX A-50 COLUMN PHOTOLYSIS LABELING EXPERIMENT. A 1-cm quartz cuvet filled with swelled DEAE-Sephadex A-50 has an observed optical density at 254 nm of 1.5, so that it was possible to photolyze I in a quartz column packed with this gel. For the columns described in the Experimental Section, the extent of photolysis depended on the flow rate, one-third of the compound being photolyzed with a flow rate of 0.5 ml/min, and one-half at 0.33 ml/min. Incomplete photolysis was necessary in order that enzyme bound to the bottom of the column have the opportunity to bind unphotolyzed derivative and become covalently labeled. The per cent of labeled enzyme formed as a function of total volume of solution containing I passed through the column is given in Table IV. The dependence of incorporation on carbene formation is demonstrated by the low level of incorporation obtained in the absence of irradiation.

Irradiation Effects. The effects of prolonged irradiation at 253.7 nm on enzymatic activity and on the ability of enzyme to incorporate I on photolysis are summarized in Table V. Other data showing changes in phosphofructokinase on photolysis are given in Table IV.

Phosphofructokinase Kinetic Studies. Figure 1 and Table VI compare the kinetic properties of native and covalently modified enzymes. Covalently modified enzyme was prepared by the column photolysis technique, was nominally labeled to the extent of 63% (although we believe this to reflect labeling of ~90% of available cAMP sites—see Discussion), and had been irradiated for 1 hr (see Table IV). This sample was used since it represented the best compromise between nearly maximal labeling and retention of enzymatic activity. The results with native enzyme are similar to what has been found previously (Uyeda and Racker, 1965;

TABLE V: Effect of Irradiation at 253.7 nm on Phosphofructokinase.

Time of Irradiation[a] (sec)	Enzyme Act.[b] (PFK Units/mg) pH 6.9[c]	pH 6.9 + cAMP (0.1 mM)[c]	pH 8.0	% cAMP Sites Labeled[e]
0	47 ± 5	206 ± 21	1040 ± 20	17.1 ± 1.0
5	71 ± 7	227 ± 23	985 ± 20	
10	95 ± 10	213 ± 21	910 ± 18	
30	135 ± 16	175 ± 18	790 ± 16	
30[d]	132 ± 13	184 ± 18		
60				17.7 ± 1.0
180				17.3 ± 1.0
600				11.6 ± 1.0

[a] Solutions were photolyzed in the Rayonet photochemical reactor. $t_{1/2}$ for photolysis of I (30 μM) was 7 ± 1 sec. [b] Enzyme (7.7 μM) dissolved in buffer A containing prephotolyzed I (20 μM) was irradiated for the times indicated and assayed. [c] Assayed as described previously (Brunswick and Cooperman, 1971). [d] Enzyme irradiated in the absence of prephotolyzed I. [e] Enzyme (30 μM) in buffer B was irradiated for the time indicated. I (290 μM) was then added and the resulting solution photolyzed to completion. Incorporation was measured by removal of noncovalently bound photolyzed I on Sephadex G-25 followed by Cl_3CCOOH precipitation as previously described (Brunswick and Cooperman, 1971).

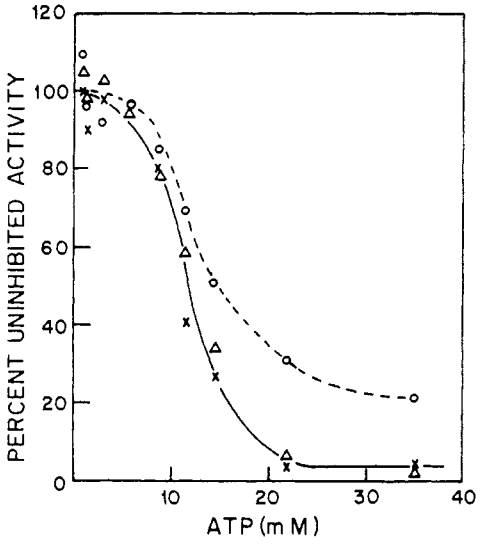

FIGURE 1: Effect of ATP on the phosphofructokinase activity of native, irradiated, and covalently modified enzymes. Assays are at pH 7.1; 30°. Mg^{2+} concentration, 50 mM: (×) native enzyme; (△) irradiated enzyme; (○) covalently modified enzyme. Data have been normalized to 100% enzyme activity at noninhibitory ATP concentrations (<2 mM). The relative activities are: native enzyme, 1.00; irradiated enzyme, 0.73; covalently modified enzyme, 0.46 (see footnote 3). Assays are accurate to ±10%.

Kemp, 1971). Since the observed differences in the properties of labeled enzyme could in principle be due either to covalent modification or to irradiation effects of the enzyme, the kinetic properties of enzyme irradiated on a DEAE-Sephadex column for 1 hr in the presence of cAMP were examined. As seen in Figure 1 and Table VI, once a correction has been made for activity lost on irradiation[3] the properties of irradiated enzyme are virtually identical with those for native enzyme. The changed properties of enzyme irradiated in the presence of I may thus be attributed to covalent modification.

Discussion

Factors Limiting Incorporation—Enrichment Methods. A common feature of studies with photoaffinity reagents has been that quantitative labeling of the target site has not been achieved (Converse and Richards, 1969; Rosenstein and Richards, 1972; Fleet *et al.*, 1972). This result can be understood in terms of the simplified scheme presented below, where E is enzyme, D is diazo compound, A is the product of reaction of photolyzed D with solvent (presumably the hydroxy compound), EA and ED are noncovalent complexes, and EA′ is the product of reaction of photolyzed D with enzyme, *i.e.*, labeled enzyme. Before photolysis, E, D,

[3] The results in Figure 1 are based on assays performed on modified, irradiated, or native enzyme after storage in buffer B, at a concentration of 3 μM, at 4° overnight. Assays performed on the same day as the modification and irradiation experiments gave qualitatively similar results, except that modified enzyme had higher activity relative to native enzyme. That is, in the plateau region observed below 2 mM ATP, at pH 7.1, on the day of the experiment the relative specific activities were 1.00, 0.82, and 0.77 for native, irradiated, and covalently modified enzymes, whereas after overnight storage they were 1.50, 1.10, and 0.69. Thus, immediately after irradiation, covalently modified and irradiated enzyme loses activity to approximately the same extent, whereas, on storage, irradiated enzyme shows an apparent gain in activity almost equal to that seen with native enzyme, while the activity of covalently modified enzyme is virtually unchanged.

TABLE VI: cAMP Reactivation of ATP-Inhibited Enzyme.[a]

[cAMP] (mM)	Relative Activities[b] Native Enzyme	Irradiated[c] Enzyme	Covalently[d] Modified Enzyme
	3.3 ± 2.0	6.7 ± 2.0	31 ± 3.1
15.6[e]	50.1 ± 5.0	49.0 ± 4.9	41.8 ± 4.2
39.1	36.7 ± 3.7	41.2 ± 4.1	38.2 ± 3.8

[a] Assays were done at pH 7.1 in the presence of 50 mM Mg^{2+} and 22 mM ATP. [b] Activity in the plateau region of the ATP curve, below 2 mM (Figure 1), taken as 100. [c] Irradiated for 1 hr on a DEAE-Sephadex column in the presence of cAMP. [d] Labeled to the extent of ~90%—see text. [e] This corresponds to a saturating concentration for cAMP reactivation. The inhibition seen at still higher cAMP concentrations may be due to a weak binding to the active site.

$$E + D \overset{K_D}{\rightleftharpoons} ED \quad (1)$$

$$E + A \overset{K_A}{\rightleftharpoons} EA \quad (2)$$

$$h\nu + D \longrightarrow A \quad (3)$$

$$h\nu + ED \longrightarrow EA \quad (4)$$

$$h\nu + ED \longrightarrow EA' \quad (5)$$

and ED are present in solution. On irradiation, D is converted into A and ED partitions into EA and EA′. In our work, photolysis has a half-life of seconds, which should be slow compared to the time needed for reequilibration among E, EA, and ED as D is converted to A. Thus, as photolysis proceeds, EA replaces ED and since EA′ can only arise from ED, labeling is incomplete. Verification that A competes with D in our work comes from experiments 1–4 in Table I. The partitioning of ED into EA and EA′ is shown by the incomplete incorporation of label into enzyme when enzyme is present in a large excess over reagent (experiment 5).

From this analysis it is clear that quantitative labeling can in principle be achieved by an iterative method which removes A from solution and introduces fresh D. In this paper we describe two continuous methods for doing this. Of the two, the column method has been found to be easier to set up, more rapid, and more reproducible and should be considered the method of choice. Its use does, however, depend on finding conditions where enzyme will stick to the column while reagent passes through, and if these conditions cannot be found then the dialysis method offers an alternative.

Radiation Damage. An inherent problem in photoaffinity reagent studies is the possibility that the receptor will be damaged by the radiation used to generate the reactive species (Knowles, 1972). In the present study two questions are of importance. First, in both continuous labeling experiments the maximum labeling obtained is 70 ± 5% of the calculated cAMP sites present. Is this due to a progressive photochemical destruction of the cAMP site, or is it due rather to an improper calculation of cAMP stoichiometry? Second, do the observed differences in kinetic behavior in covalently modified and native enzyme arise entirely as a

consequence of covalent binding, or do radiation effects on the enzyme also have to be considered?

We believe our failure to achieve 100% labeling is due to an inaccurate calculation on the following grounds. (a) No decrease in ability to incorporate I on photolysis is seen for enzyme preirradiated for a time equivalent to 25 half-lives for I photolysis (Table V), although an effect is seen on preirradiation for a much longer time. The incorporation of I into enzyme irradiated on a column for 20 half-lives of I photolysis is already very close to the maximal labeling achieved (Table IV). This is consistent with results presented in Table II, which show substantial increases in incorporation even after prolonged irradiation. (b) Our calculation assumes we have 100% pure, active enzyme, although in fact we use a commercial preparation, which, in preliminary experiments on an AMP affinity column (K. Nealy, unpublished observations), was shown to contain about 20–30% (estimated by $A_{280\,nm}$) of a protein lacking phosphofructokinase activity. Moreover, we have found that different samples of enzyme obtained from Sigma incorporate different amounts of I (per $A_{280\,nm}$ unit) under identical experimental conditions. For example, in simple photolysis experiments saturated levels of incorporation have varied from 23 to 35% on different preparations of enzyme. Similarly, aged samples of enzymes show reduced incorporation levels, although there is no obvious correlation of incorporation with specific activity measured at pH 8. For these reasons our calculations of per cent of sites labeled, while useful to demonstrate saturation or enrichment (Tables II and IV), are accurate only as relative values, and we believe it probable that the maximum values obtained in Tables II and IV represent quantitative labeling of available cAMP sites.

With respect to the second question, our control irradiation experiment is good evidence that irradiation by itself has little effect on ATP inhibition and partial cAMP reactivation measured at pH 7.1. However, the kinetic behavior of phosphofructokinase is exceedingly complex, and assays under other conditions could well show important changes on irradiation [see, for example, the dramatic increase in activity at pH 6.9 (Table V)].

Phosphofructokinase Kinetics. Mathias and Kemp (1972) have previously presented evidence that phosphofructokinase has two, noncatalytic sites for nucleotides. The first, an activator site, has a high affinity for cAMP and little or no affinity for ATP, while the second, an inhibitor site, has a high affinity for ATP and very low affinity for cAMP. Our kinetic studies, showing that ATP substantially inhibits a sample of enzyme whose cAMP sites are almost entirely blocked, and does so with an apparent affinity virtually the same as that for native enzyme (Figure 1), are fully in accord with this model.

More detailed interpretation of our results would obviously require more quantitative data on the extent of labeling and of the effects of irradiation. However, a more fundamental and difficult problem for detailed interpretation is that posed by the nonselectivity of carbene insertion—that is, since incorporation might well take place into several different amino acid residues at the binding site (Hexter and Westheimer, 1971) there exists the very real possibility that "covalently modified" enzyme is actually a mixture of products, each having somewhat different kinetic properties. In our present ignorance of both the identity and possible multiplicity of insertion products, only qualitative conclusions of the kind made above are worthwhile.

Irradiation at 253.7 nm: A General Technique? It is important to consider whether the rather minor changes on 253.7-nm irradiation seen with phosphofructokinase represent typical or extraordinary behavior, in order to assess whether the method described in this paper will be applicable to other enzymatic studies. In Table VII we compare the calculated relative rates of photolysis at 253.7 nm of ethyl-2-diazomalonic acid and of the amino acid residues and peptide backbone of phosphofructokinase. The rates are calculated as the product of the extinction coefficients, quantum yields, and stoichiometry per protomer following the procedure suggested by Luse and McLaren (1963). Although this procedure depends on several questionable assumptions, it has been applied with approximate success to other enzymes (McLaren, 1969) and can provide some qualitative guidance. The total calculated rate for a single photolytic reaction in phosphofructokinase is about 10% of the rate of photolysis of the ethyldiazomalonyl group. As not every photolytic reaction of protein would be expected to lead to a significant change in properties, the rate of functional change of phosphofructokinase should be less than one-tenth that of carbene formation from I. Examination of Table V indicates that, for at least some properties, this prediction is borne out. However, the activation at pH 6.9 is clearly proceeding more rapidly than predicted from Table VII. Overall, phosphofructokinase may not be atypical of enzymes. The lack of S–S bonds is certainly desirable for photolysis studies, but this is balanced by the rather high equivalent weight for cAMP binding. Tryptophan residues, which are especially sensitive to photolysis, constitute 1.3% of the residues in phosphofructokinase (Paetkau *et al.*, 1968), which is close to the average value for 108 proteins (1.2%) listed by Dayhoff and Hunt (1972).

I is typical of diazo ketones in having two absorption bands in the ultraviolet, one intense (λ_{max} 254 nm, ϵ_{max} 7000)[4] and one rather weak (λ_{max} 350 nm, ϵ_{max} ~10), and carbenes may be generated by irradiation at either wavelength. We have used the shorter wavelength in these studies because of our previous results showing that irradiation at 350 nm gave only 25% as much incorporation as irradiation at 253.7 nm (Brunswick and Cooperman, 1971) and because of the rela-

TABLE VII: Photolysis of Amino Acids, Peptide Linkages, and Ethyl-2-diazomalonic Acid by Irradiation at 253.7 nm.[a]

Species	$\epsilon_{253.7}$	ϕ[b]	$\epsilon\phi$	$n\epsilon\phi$[c]
Cystine	270	0.13	35.1	0
Histidine	0.24	<0.03	<0.007	<0.14
Phenylalanine	140	0.013	1.8	54.9
Tryptophan	2870	0.004	11.5	140
Tyrosine	320	0.002	0.64	11.4
-CONH-	1.0	0.005	0.005	4.6
Total for phosphofructokinase				211
Ethyl-2-diazomalonic acid	7000	~0.3[d]	2100	2100

[a] Data for amino acids and peptide linkages from McLaren and Shugar (1964). [b] Quantum yield. [c] n is the number of residues per protomer of phosphofructokinase (Paetkau *et al.*, 1968). [d] Estimated on the basis of values for other α-diazo-β-dicarbonyl compounds (Calvert and Pitts, 1966).

[4] This value is obtained by subtracting out the purine spectrum from the observed spectrum of I.

tive insensitivity of the enzyme to 253.7-nm irradiation. For more sensitive enzymes 350-nm irradiation might be more appropriate. However, it is well to point out that carbenes generated at this wavelength are less reactive (Vaughan, 1970) and thus less desirable as labeling reagents than those generated at 253.7 nm. Morever, for photolysis to proceed at a reasonable rate, a quite intense light source would be needed. Radiation damage of fully labeled enzyme might also be minimized by irradiation of enzyme for shorter periods than those used in this paper followed by resolution of labeled and unlabeled enzyme through use of affinity chromatography (Cuatrecasas and Anfinsen, 1971). This approach is currently being pursued.

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