ROSE BENGAL IMMOBILIZED ON SEPHAROSE – A NEW TOOL FOR PROTEIN PHOTO-OXIDATION

N. M. C. KAYE and P. D. J. WEITZMAN
Department of Biochemistry, University of Leicester, Leicester, England

Received 24 December 1975

1. Introduction

In a previous report [1] we described the unusual response of *Acinetobacter lwoffi* citrate synthase (EC 4.1.3.7) to dye-sensitized photo-oxidation. The novelty of these findings lay in the complementary pattern of pH dependence of photo-inactivation of the enzyme observed with the cationic dye, Methylene Blue, and the anionic dye, Rose Bengal. With Methylene Blue, the rate of photo-inactivation increased with increasing pH, whereas with Rose Bengal the rate increased with decreasing pH over the range of pH 8 to 5. This abnormal behaviour with Rose Bengal prompted the suggestion that binding of the dye to the enzyme was necessary for the oxidation.

Subsequent studies [2] have revealed that citrate synthases from other sources show essentially identical behaviour on photo-oxidation to that of the *Acinetobacter* enzyme. In an attempt to investigate the involvement of dye-enzyme binding in the photo-oxidation process it seemed to us that the use of Rose Bengal immobilized on an insoluble matrix might be helpful in preventing or reducing the interaction between dye and enzyme. More generally, the use of matrix-immobilized Rose Bengal for the photo-oxidation of proteins could offer some practical advantage over the free-solution form of the dye. In particular, removal of the dye from the photo-oxidized protein, a necessary step prior to further study of the modified protein or determination of its aminoacid composition, could be rapidly effected by simple filtration or centrifugation. This would contrast favourably with the more laborious conventional procedures of gel filtration, dialysis or treatment with charcoal.

In this communication we describe the simple preparation of Rose Bengal immobilized on AH-Sepharose (the commercially available aminohexyl derivative of Sepharose) and present data to show its efficacy in bringing about the photo-oxidation of the five photo-sensitive aminoacids as well as of the enzyme citrate synthase. It is suggested that this immobilized form of Rose Bengal may prove of general usefulness in photo-oxidation studies.

2. Experimental

Rose Bengal was obtained from BDH Chemicals Ltd. and was further purified by the method of Brand et al. [3]. Its purity was confirmed by the observation of a single spot on thin-layer chromatography performed according to [3] or [4]. Sepharose 4B, AH-Sepharose 4B and CH-Sepharose 4B were from Pharmacia Fine Chemicals, high grade L-aminoacids from BDH Chemicals Ltd., 1-ethyl-3(3-dimethylaminopropyl)carbodiimide from Sigma London Chemical Co. Ltd., and pig heart citrate synthase from Boehringer Corporation (London) Ltd.

Assays of citrate synthase activity were performed spectrophotometrically at 412 nm [5] in 0.1 M Tris-HCl, pH 8.0, with 0.2 mM oxaloacetate, 0.15 mM acetyl-CoA and 0.1 mM 5,5′-dithiobis-(2-nitrobenzoate).

Rose Bengal was immobilized on AH-Sepharose 4B in the following way. AH-Sepharose 4B (0.5 g of freeze-dried powder) was allowed to swell in an excess of 0.5 M NaCl solution for 15 min. It was

 North-Holland Publishing Company – Amsterdam

then collected on a sintered glass funnel and washed with 200 ml of 0.5 M NaCl, followed by 200 ml of distilled water. The gel was transferred to a beaker with 10–20 ml of water and 0.5 ml of 1 mM Rose Bengal (in water) was added. Binding of the dye to the gel appeared to take place instantly and completely. The AH-Sepharose–Rose Bengal complex was washed on a sintered glass funnel with, successively, 0.1 M K_2HPO_4/0.5 M NaCl, 0.1 M KH_2PO_4/0.5 M NaCl, and, finally, distilled water. Negligible removal of dye was detected. Knowing the amount of Rose Bengal used, the washed product was suspended in a volume of water calculated to give the particular dye concentration required.

The procedures used to follow the progress of photo-oxidation were as follows. In the case of the free aminoacids, photo-oxidation was followed by measuring the oxygen uptake manometrically. A circular transparent plastic water-bath was maintained at 10°C with a copper cooling coil supplied with water from a refrigerated source. Photo-oxidation reactions were carried out in Warburg flasks positioned in this water-bath and shaken at 86 strokes/min with an amplitude of 7 cm. Each flask was irradiated with a 100 W spot-light placed 16 cm from the mean position of the flask. The flasks were prepared by introducing 1.65 ml of 20 mM Tris-HCl, pH 8.0, containing 0.05 μmol of Rose Bengal, into the main cup. Into the side-arm were placed 0.35 ml of solution containing 5 μmols of aminoacid in 20 mM Tris-HCl adjusted to pH 8.0, while the centre well contained 0.2 ml of 20% KOH with a filter paper wick to absorb any CO_2 evolved during photo-oxidation. After temperature equilibration had been attained the vessels were closed and shaking was continued for a further 5 min to confirm true equilibration. The aminoacid solution was then tipped from the side-arm into the main cup and the light switched on. Oxygen uptake was measured over a period of 20 min, during which time the rate of uptake (μl O_2/min) was constant in all cases. Control experiments were carried out in the absence, respectively, of dye, light or aminoacid, but no oxygen uptake was observed.

In the case of citrate synthase (pig heart) photo-oxidation was followed by its inactivating effect on the enzyme. A 100 W spot-light was positioned 14 cm from the reaction mixture contained in a conical glass tube surrounded by a water-jacket through which was circulated refrigerated water at 6°C. Into the tube were placed 0.4 ml of buffer solution (50 mM phosphate at various pH values from 5 to 8) and 0.05 ml of 30 μM Rose Bengal (either free or immobilized). The light was switched on and 0.05 ml of enzyme solution (10 μg of protein) added and mixed. At 2-min intervals over a period of 12 min, 0.05 ml samples were withdrawn and immediately diluted five times with 0.1 M Tris-HCl, pH 8, in small glass tubes covered with aluminium foil, to prevent further reaction. Suitable control experiments were carried out in the absence of dye or light. At the lower pH values examined, control rates of enzyme inactivation in the absence of dye were subtracted from the observed photo-inactivation rates.

3. Results and discussion

We first attempted to bind Rose Bengal to AH-Sepharose 4B by the carbodiimide procedure, aiming to link the carboxyl group of the dye to the amino group of the Sepharose derivative. After addition of Rose Bengal to the swollen gel the pH was adjusted to 4.7 and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide was added to a final concentration of 0.1M. The pH was maintained at 4.7 with HCl and the mixture was left overnight at room temperature and then thoroughly washed. However, in the course of this preparation it appeared that strong binding of the Rose Bengal to the AH-Sepharose occurred even before the addition of carbodiimide and, as reported under Experimental, no dye could be removed from the AH-Sepharose-Rose Bengal complex prepared by simple mixing in the absence of carbodiimide. We tested the photo-oxidizing capacity of dye–Sepharose complex prepared with and without carbodiimide and found the two preparations to behave identically. It is therefore sufficient simply to mix Rose Bengal and AH-Sepharose in order to obtain a tightly bound complex.

No binding of Rose Bengal to ordinary Sepharose 4B or to CH-Sepharose 4B (containing hexanoic acid groups) was observed either on simple mixing or after reaction with carbodiimide. There thus appears to be a very strong and specific binding of Rose Bengal to AH-Sepharose which presumably resides in very strong polar interaction between the anionic dye and

Table 1
Photo-oxidation of amino acids

Amino acid	Rate of O_2 uptake (μl/min)	
	Free dye	Immobilized dye
Cysteine	3.84	0.74
Histidine	2.04	0.51
Methionine	2.56	0.38
Tryptophan	1.69	0.34
Tyrosine	1.79	0.34
Alanine	0	0
Glutamate	0	0

Photo-oxidation was carried out at pH 8.0 with either free or immobilized Rose Bengal (25 μM) and was followed manometrically as described in the text.

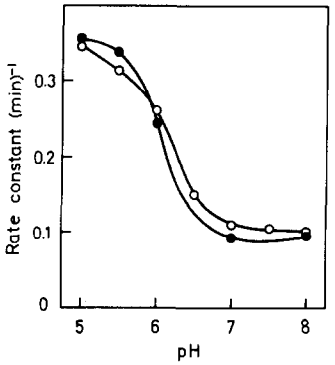

Fig.1. Photo-oxidation of citrate synthase. Photo-oxidation was carried out at various pH values with either free (○) or immobilized (●) Rose Bengal (3 μM) as described in the text. The apparent first-order rate constants were determined from the slopes of semi-log plots of the decay of enzymic activity against time of photo-oxidation.

the cationic amino groups on the AH-Sepharose, though other types of interaction may also play a contributory role.

Table 1 shows the rates of oxygen uptake observed on photo-oxidation of aminoacids with both free and immobilized forms of Rose Bengal. Although the rates of oxygen uptake with the immobilized dye are about 20% of those with the free dye (at the same total dye concentration) nevertheless the immobilized dye can clearly effect photo-oxidation of the five aminoacids known to be susceptible to attack. As exemplified by alanine and glutamate, no photo-oxidation occurs with other aminoacids.

The results of photo-oxidizing pig heart citrate synthase with either free or immobilized Rose Bengal are shown in fig.1. It is seen that the pH dependence of photo-inactivation of this enzyme is similar to that previously reported for *Acinetobacter* citrate synthase [1]. Significantly, the pattern of photo-oxidation is essentially the same whether the free or immobilized dye is used, and the actual rates of photo-inactivation are very similar for the two forms of the dye. Thus, although free aminoacids appear to be photo-oxidized more slowly with the immobilized dye than with the free dye, this particular protein, at least, reacts with comparable sensitivity.

We therefore suggest that immobilized Rose Bengal may be a useful tool in the photo-oxidative modification of other proteins. In the particular experiments reported here we did not take advantage of the easier removal of immobilized dye from reaction mixtures but treated reaction mixtures with free or immobilized dye in the same way in order to obtain the strictest comparison between the two forms. In other experiments we have found the total removal of immobilized dye by a very brief microcentrifugation to be an immense advantage.

Other workers have reported the immobilization of Rose Bengal on insoluble supports, e.g. chloromethylated styrene–divinylbenzene copolymer beads [6] and silica gel [7] and have used these forms of the dye to investigate the photo-oxidation of various organic compounds. The method of immobilization which we report here is considerably simpler than other procedures and the use of Sepharose as the supporting matrix is particularly suitable for aqueous reaction mixtures. Moreover, our demonstration of the effectiveness of the Sepharose-bound dye in photo-oxidizing all five susceptible aminoacids and in photo-inactivating an enzyme suggests that this particular form of immobilized Rose Bengal may find more general application

Finally it should be noted that the anomalous pH dependence of photo-oxidation of citrate synthase with Rose Bengal [1] has not been altered by immobilization of the dye. Were binding of the dye to the enzyme to be responsible for the unusual pH dependence, as was suggested [1], one might have anticipated some effect arising from a restriction of this binding by the proximity of the Sepharose matrix.

The absence of any alteration and its significance for the mechanism of photo-oxidation, as well as the application of immobilized Rose Bengal to other studies, are currently being investigated.

Acknowledgements

We thank the Science Research Council for support (Grant B/RG/45952) and for a Research Studentship to N.M.C.K.

References

[1] Weitzman, P. D. J., Ward, B. A. and Rann, D. L. (1974) FEBS Lett. 43, 97–100.
[2] Kaye, N. M. C. and Weitzman, P. D. J., in preparation.
[3] Brand, L., Gohlke, J. R. and Rao, D. S. (1967) Biochemistry 6, 3510–3518.
[4] Biorck, G., Gardell, S., Carlberger, G. and Meurman, L. (1960) Nature 185, 847–848.
[5] Srere, P. A., Brazil, H. and Gonen, L. (1963) Acta Chem. Scand. 17, S129–S134.
[6] Schaap, A. P., Thayer, A. L., Blossey, E. C. and Neckers, D. C. (1975) J. Amer. Chem. Soc. 97, 3741–3745.
[7] Nilsson, R. and Kearns, D. R. (1974) Photochem. Photobiol. 19, 181–184.