Diagnostic Test for Ruthenium and Platinum Modification of Histidine Residues on Metalloproteins Using Diethylpyrocarbonate (DEPC)

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Abstract

The reaction of diethylpyrocarbonate (DEPC) with uncoordinated surface histidine residues on metalloproteins at pH 6.5–7.0 can be readily monitored (~30 min) by spectrophotometric changes at ~238 nm ($\epsilon = 2750$ M^{-1} cm^{-1}). No reaction is observed if prior modification of the histidine by attachment of Ru(II) or Pt(II) has been carried out. In the studies with azurin and cytochrome *c* only one of two histidines readily undergoes DEPC modification, and likewise only one histidine is Ru modified. Thus the reaction can be used as a quick test for histidine availability, and subsequently as a test as to whether modification has been achieved. The specificity of Pt complexes for histidines is less than that of Ru. In a wider context of histidine availability only one of three histidines in Rieske's protein (*N. crassa*) is DEPC modified.

Introduction

The reaction of diethyl pyrocarbonate (DEPC) with imidazole and substituted imidazoles (including histidine) results in the formation of *N*-carbethoxy derivatives which are reported to absorb strongly in the UV region ($\lambda_{max} \sim 240$ nm, $\epsilon_{max} \sim 3200$ M^{-1} cm^{-1}) [1]. Although the reagent does not always react exclusively with the histidine residues on proteins, it is nevertheless more selective than any other acylating agent, especially if experimental conditions such as pH and reagent excess are carefully controlled. Consequently, it has been extensively used in the modification of histidine residues on proteins, to provide information on the role of specific histidines in determining the activity of enzymes [2–5], the mechanism of electron-transfer reactions [6–8], and the pH-dependence of protein NMR spectra [9].

Recent interest in attaching inorganic redox partners (principally ruthenium derivatives) to metalloproteins [10–17], in order to study fixed distance electron-transfer processes, has prompted us to investigate the reaction of DEPC with a number of metalloproteins in order to assess its potential usefulness as a facile and rapid means of determining the number of modificable histidines on a given protein under specified conditions, and more importantly as a means of confirming the actual attachment of the metal ion to the targeted histidine.

Experimental

The source and/or isolation procedures for the different metalloproteins were as follows: *Scenedesmus obliquus* plastocyanin (from Dr. R. Powls, University of Liverpool), *Anabaena variabilis* (grown in 20 l vessels) plastocyanin, high potential iron–sulphur protein (HIPIP) from *Chromatium vinosum* (cell paste from PHLS, Porton Down, U.K.), *Pseudomonas stutzeri* (grown in a 20L LH Fermentor) cytochrome c_{551}, *Pseudomonas aeruginosa*, (cell paste from PHLS) azurin, horse-heart cytochrome *c* (Sigma Chemicals), [18–23]. The reaction of Rieske's iron sulphur protein from *Neurospora crassa* (Professor H. Weiss, Universitat Dusseldorf) was also studied [24].

The Ru(II) and Pt(II) complexes used in protein modification procedures were prepared by literature methods: $[Ru(NH_3)_5H_2O](PF_6)_2$, $[Pt(NH_3)_3Cl]Cl$ and $[Pt(terpy)Cl]Cl \cdot 2H_2O$, where terpy is the tridentate ligand, 2,2′-6′,2″-terpyridine (Sigma Chemicals). Ruthenium-modification *i.e.* $Ru(NH_3)_5$ attachment, to two plastocyanins, HIPIP, cytochrome c_{551}, and to azurin, and Pt-modification of cytochrome *c* (horse-heart) by $[Pt(NH_3)_3Cl]Cl$ and $[Pt(terpy)Cl]Cl$ are considered here [18–32]. All other chemicals used were of Analar quality.

Stock solutions (0.2 M) of diethylpyrocarbonate, DEPC (Sigma Chemicals), in ethanol were stored at 4 °C under N_2, and standardised before use against solutions of imidazole, (in 0.10 M phosphate buffer at pH 6.5–7.0). In a typical experiment, two narrow quartz cells of 1 cm path length were filled with equal

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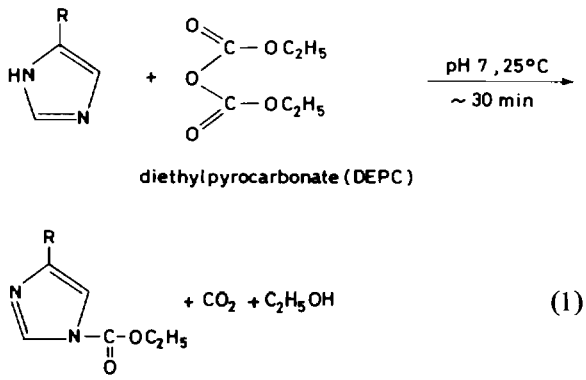
volumes (1.3 ml) of protein (0.5 mg) in phosphate buffer, and placed in the sample and reference compartments (25 °C) respectively of a spectrophotometer (Perkin-Elmer 554). A ~0.2 M ethanol solution of DEPC (5 μl) was introduced into the sample cell, while an equal volume of ethanol was added to the reference. After thorough mixing the solutions were scanned repeatedly over the range 220–300 nm at appropriate time intervals until no further change of absorbance was observed.

Results and Discussion

The reaction of DEPC with imidazoles is as indicated in eqn. (1)



(1)

In the presence of a very large excess (>200 fold) of DEPC, reaction at the second N-atom of the imidazole ring is observed. It has been reported that DEPC can also react with lysine, tyrosine, serine and thiolate groups of proteins [1]. However, such reactions are usually restricted to acidic solutions (pH ~ 4). The pH and other experimental conditions used were such as to minimise reactions with other amino acids, and maximise stability of the proteins. Furthermore, the *N*-carbethoxy derivatives formed are kinetically stable at pHs in the range 6.5–7.0. The DEPC excess per histidine residue (15–25 fold) was sufficient to ensure completion of eqn. (1), but insufficient for the second reaction to occur.

Figure 1A shows the spectrophotometric trace of the reaction of DEPC with plastocyanin (*S. obliquus*), which is typical of the reactions of DEPC with the metalloproteins listed in Table I. The number of histidine residues modified can be calculated from the absorbance maximum at ~240 nm using the absorption coefficient $\epsilon = 3200\ M^{-1}\ cm^{-1}$ reported for the carbethoxy derivative of imidazole [1]. It should be noted that for all the proteins listed in Table I, on addition of DEPC the absorbance changes are at 238 nm. Also the number of histidines modified, based on $\epsilon = 3200\ M^{-1}\ cm^{-1}$, are 80–90% of the values expected. Absorption coefficients for the carbethoxy derivatives of histidine residues in proteins are consistently less than that for imidazole alone [1]. Tudball *et al.* have reported a value of $\epsilon = 2900\ M^{-1}\ cm^{-1}$ at pH 7.5 for the *N*-ethoxyhistidine protein derivative [5], and Nettesheim *et al.* have reported a value 90% of 3200 $M^{-1}\ cm^{-1}$ for the modification of HIPIP (*C. vinosum*) [7]. The values listed in Table I have been rounded off to the nearest integer, using $\epsilon = 2750$. An examination of Table I reveals that under the experimental conditions not all of the uncoordinated histidine residues undergo reaction with DEPC. With azurin, for example, there are two uncoordinated histidine residues, yet only one of these is modified Fig. 1B.

When singly Ru-modified *S. obliquus* plastocyanin is treated with DEPC, no absorbance changes at 238 nm characteristic of *N*-carbethoxy modification of the sole uncoordinated histidine (His59) are observed. Protein modification by attachment of $Ru(NH_3)_5^{3+}$ to the histidine completely blocks reaction with the

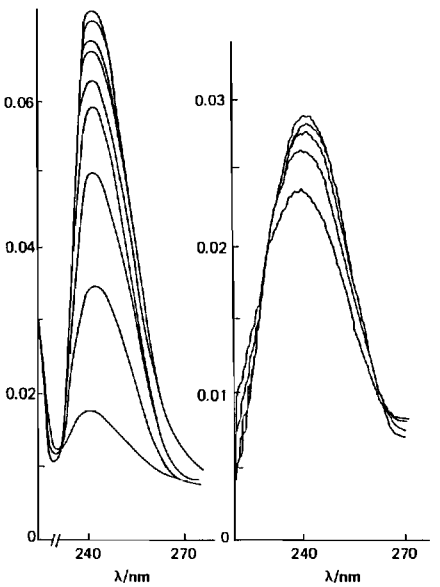


Fig. 1. (A) The reaction of diethylpyrocarbonate (DEPC) (360 μM) with native *S. obliquus* plastocyanin (24 μM) at pH 7.0 (0.10 M phosphate buffer) at 25 °C. (B) The reaction of diethylpyrocarbonate (DEPC) (120 μM) with *P. aeruginosa* azurin (8 μM) at pH 6.5 (0.10 M phosphate buffer) at 25 °C. Scans were recorded every 5 min, final scan after a further 15 min. For B the final absorbance at 238 nm indicates that only one of two uncoordinated histidines is modified.

TABLE I. Summary of Results for Diethylpyrocarbonate (DEPC) Modifications at pH 6.5–7.0[a]

Protein	Number of uncoordinated histidine residues in protein	Number of histidines modified by DEPC	Number of histidines modified by DEPC in Ru/Pt-modified protein
Plastocyanin (*S.o.*)	1	1	0
Plastocyanin (*A.v.*)	1	1	0
HIPIP (*C.v.*)	1	1	0
Cytochrome c_{551} (*P.s.*)	1	1	0
Azurin (*P.a.*)	2	1	0
Cytochrome *c* (h.h.)	2	1	0[b] and 1[c]

[a]Reaction conditions as indicated in text, duration of modification generally ~30 min. [b]Pt-modified derivative: attachment of $Pt(terpy)^{2+}$ is at His33. [c]Pt-modified derivative: for one of the products attachment of $Pt(NH_3)_3^{2+}$ is not at His33.

DEPC. Similar effects are observed with *A. variabilis* plastocyanin (His59), HIPIP from *C. vinosum* (His42), and cytochrome c_{551} from *P. stutzeri* (His47), the native forms of which all give spectrophotometric changes consistent with DEPC modification of the single available histidine, Table I. For the Ru-modified derivatives, attachment of $Ru(NH_3)_5^{3+}$ to the histidines in question has been further confirmed by NMR studies.

In the case of *P. aeruginosa* azurin (His35 and 83) and horse-heart cytochrome *c* (His26 and 33), the major products each contain Ru attached to a single histidine residue. The singly-modified Ru derivative of azurin does not react further with DEPC. The availability or non-availability of histidines for DEPC modification appears to apply also to their availability for Ru-modification.

The reaction of horse-heart cytochrome *c* with platinum complexes further illustrates the utility of this reagent as a rapid indicator of site of attachment. Reaction with $[Pt(NH_3)_3Cl]^+$ yields two products in approx. equal amounts, one of which gives a reaction with DEPC. The other does not. In the former case therefore Pt modification is not at His33. However, use of $[Pt(terpy)Cl]^+$ results in the formation of a singly modified protein that does not undergo reaction with DEPC, and the site of attachment of the Pt is confirmed as His33 [31]. While the site of modification in the case of $[Pt(NH_3)_3Cl]^+$ is in this case His33, in the other it is probably at Met-65, by analogy with the reaction of $[PtCl_4]^{2-}$ with cytochrome *c* for which attachment is 10% at His33 and 90% at Met65 [33–34].

In further experiments we have found that only one of three histidines in Rieske's protein from *N. crassa* (25 μM) is modified by DEPC treatment, which may implicate the remaining two histidines in binding to the [2Fe–2S] active site, as recently proposed for Rieske's protein from *Thermus thermophilus* [35].

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References

1 E. W. Miles, *Methods Enzymol., 47,* 431 (1977).
2 H. Kumagai, T. Utagawa and H. Yamada, *J. Biol. Chem., 250,* 1661 (1975).
3 Y. Burstein, K. A. Walsh and H. Neurath, *Biochemistry, 13,* 205 (1974).
4 W. B. Melchior Jr. and D. Fahrney, *Biochemistry, 9,* 251 (1970).
5 N. Tudball, R. Bailey-Wood and P. Thomas, *Biochem. J., 129,* 419 (1972).
6 D. J. Davis and B. J. Vieira, *Biochem. Biophys. Res. Commun., 112,* 508 (1983).
7 D. G. Nettesheim, W. V. Johnson and B. A. Feinberg, *Biochim. Biophys. Acta, 593,* 371 (1980).
8 P. L. Drake, R. T. Hartshorn, J. McGinnis and A. G. Sykes, to be published.
9 F. A. Leitch, G. R. Moore and G. W. Pettigrew, *Biochemistry, 23,* 1831 (1984).
10 M. P. Jackman, A. G. Sykes and G. A. Salmon, *J. Chem. Soc., Chem. Commun.,* 65 (1987).
11 S. S. Isied, C. Kuehn and G. Worosila, *J. Am. Chem. Soc., 106,* 1722 (1984).
12 R. Bechtold, M. B. Gardineer, A. Kazmi, B. Van Hemelryck and S. S. Isied, *J. Phys. Chem., 90,* 3800 (1986).
13 D. G. Nocera, J. R. Winkler, K. M. Yocom, E. Bordignon and H. B. Gray, *J. Am. Chem. Soc., 106,* 5145 (1984).
14 R. Margalit, N. M. Kostic, C.-M. Che, D. F. Blair, H.-J. Chiang, I. Pecht, J. B. Shelton, J. R. Shelton, W. A. Schroeder and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A., 81,* 6554 (1984).

15 K. M. Yocom, J. B. Shelton, J. R. Shelton, W. A. Schroeder, G. Worosila, S. S. Isied, E. Bordignon and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A., 79,* 7052 (1982).
16 C.-M. Che, R. Margalit, H.-J. Chiang and H. B. Gray, *Inorg. Chim. Acta, 135,* 33 (1987).
17 M. P. Jackman, M.-C. Lim, A. G. Sykes and A. G. Salmon, *J. Chem. Soc., Chem. Commun.,* 179 (1988).
18 K. Ugurbill, R. S. Norton, A. Allerhand and R. Bersohn, *Biochemistry, 16,* 886 (1977).
19 D. L. Brautigan, S. Ferguson-Miller and E. Margoliash, *Methods Emzymol., 53,* 128 (1978).
20 M.-Y. Liu, M.-C. Liu, W. J. Payne, H. D. Peck, Jr. and J. Le Gall, *Curr. Microbiol., 9,* 87 (1983).
21 R. G. Bartsch, *Methods Enzymol., 23,* 644 (1971) and refs. therein; I. K. Adzamli, D. M. Davies, C. S. Stanley and A. G. Sykes, *J. Am. Chem. Soc., 103,* 5543 (1981).
22 P. Rowell and R. Powls, *Biochim. Biophys. Acta, 423,* 65 (1976).
23 M. P. Jackman, J. D. Sinclair-Day, M. J. Sisley and A. G. Sykes, *J. Am. Chem. Soc., 109,* 6443 (1987).
24 Y. Li, S. De Vries, K. Leonard and H. Weiss, *FEBS Lett., 135,* 277 (1981); U. Harnisch, H. Weiss and W. Seebald, *Eur. J. Biochem., 149,* 95 (1985).
25 R. W. Callahan, G. M. Brown and T. J. Meyer, *Inorg. Chem., 14,* 1433 (1975).
26 H. Morita and J. C. Bailar, Jr., *Inorg. Synth., 22,* 124 (1983).
27 M. Howe-Grant and S. J. Lippard, *Inorg. Synth., 20,* 101 (1980).
28 M. P. Jackman, J. McGinnis, R. Powls, G. A. Salmon and A. G. Sykes, *J. Am. Chem. Soc., 110,* 5880 (1988).
29 M. P. Jackman, M.-C. Lim, G. A. Salmon and A. G. Sykes, *J. Chem. Soc., Dalton Trans.,* (1988) in press.
30 P. Osvath, G. A. Salmon and A. G. Sykes, *J. Am. Chem. Soc.,* (1988) in press.
31 M. P. Jackman, *Ph.D. Thesis,* University of Newcastle upon Tyne, 1987.
32 E. M. A. Ratilla, H. M. Brothers II and N. M. Kostic, *J. Am. Chem. Soc., 109,* 4592 (1987).
33 R. E. Dickerson, D. Eisenburg, J. C. Varnum and M. L. Kopka, *J. Mol. Biol., 45,* 77 (1969).
34 A. P. Boswell, G. R. Moore and R. J. P. Williams, *Biochem. J., 201,* 523 (1982).
35 J. A. Fee, K. L. Findling, T. Yoshida, R. Hille, G. E. Tarr, D. O. Hearshen, W. R. Dunham, E. P. Day, T. A. Kent and E. Munck, *J. Biol. Chem., 259,* 124 (1984).