Biochimica et Biophysica Acta, 680 (1982) 290–296
Elsevier Biomedical Press

BBA 41089

THE ROLE OF A CYTOCHROME *c*-552-CYTOCHROME *c* COMPLEX IN THE OXIDATION OF SULFIDE IN *CHROMATIUM VINOSUM*

GARY O. GRAY and DAVID B. KNAFF

Department of Chemistry, Texas Tech University, Lubbock, TX 79409 (U.S.A.)

(Received November 16th, 1981)

Key words: Cytochrome c; Sulfide oxidation; Bacterial photosynthesis; Cytochrome c-552; (C. vinosum)

The sulfide:cytochrome *c* oxidoreductase activity of the flavocytochrome *c*-522 from the purple sulfur bacterium *Chromatium vinosum* has been investigated. The oxidized sulfur product of the sulfide:cytochrome *c* reductase activity has been shown to be elemental sulfur. Cytochrome *c*-552 has been found to form a stable complex with horse heart cytochrome *c* that appears to be held together by electrostatic interactions. The stability of this complex and the sulfide:cytochrome *c* reductase activity of cytochrome *c*-552 are both ionic strength dependent, with maximal rates of cytochrome *c* reduction and extent of complex formation occurring over the same ionic strength range. Trifluoroacetylated cytochrome *c* is not reduced in the presence of cytochrome *c*-552 and sulfide, nor does it form a complex with cytochrome *c*-552. These results suggest the possible involvement of cytochrome *c* lysine residues in complex formation. Cytochrome *c*-552 migrates with an anomalously high apparent molecular weight on gel filtration columns equilibrated with low ionic strength buffers, suggesting the possibility of conformational changes or dimerization of the protein. However, complexation of cytochrome *c*-552 with cytochrome *c* still occurs at low ionic strength.

Introduction

The photosynthetic purple sulfur bacterium *Chromatium vinosum* obtains reducing equivalents through the photosynthetic oxidation of thiosulfate and sulfide [1,2]. Cells grown on thiosulfate and/or sulfide have been shown to transiently accumulate elemental sulfur, although sulfate is the ultimate oxidized sulfur-containing species [3]. While considerable information is available about portions of these pathways in both purple and green photosynthetic sulfur bacteria [4], the details of the pathway(s) by which sulfide is oxidized to sulfur in purple sulfur bacteria are unclear.

Among the large number of soluble *c*-type cytochromes found in *C. vinosum* is the flavocytochrome *c*-552 [5]. Fukumori and Yamanaka [6] have recently demonstrated that this flavocytochrome exhibits sulfide:cytochrome *c* oxidoreductase activity and have suggested that the physiological role of cytochrome *c*-552 may be to transfer electrons from sulfide to the main photosynthetic electron-transport chain through a *c*-type cytochrome. A likely candidate for the in vivo *c*-type cytochrome acceptor of electrons from sulfide in the cytochrome *c*-552-catalyzed reaction in *C. vinosum* is cytochrome *c*-551 [7–9]. Cytochrome *c*-551, located in the periplasmic space of *C. vinosum* [8], appears to be similar to horse heart cytochrome *c* in that either the endogenous *C. vinosum* cytochrome *c*-551 or horse heart cytochrome *c* will reconstitute cyclic electron transport in spheroplasts obtained from *C. vinosum* [9]. Van Grondelle et al. [8] have

Abbreviation: Tricine, *N*-tris(hydroxylmethyl)methylglycine.

0005-2728/82/0000-0000/$02.75

estimated that *C. vinosum* cytochrome *c*-551 has a midpoint oxidation-reduction potential (+260 mV) similar to that of horse heart cytochrome *c* [10]. Van Grondelle et al. have also demonstrated that cytochrome *c*-551 can donate electrons to the reaction center bacteriochlorophyll of *C. vinosum* via the membrane-bound cytochrome *c*-555 [8]. Thus, it appears that the sulfide:cytochrome *c* oxidoreductase system may serve as an excellent in vitro model for electron transfer from sulfide to the photosynthetic electron-transport chain in *C. vinosum* cells.

Horse heart cytochrome *c* is known to interact electrostatically with other electron-transfer proteins to form catalytically active complexes. Complexes involving sulfite oxidase, cytochrome oxidase, Complex III and cytochrome c_1 have been characterized [11–13]. Generally, the electrostatic interactions that form these complexes have been shown to involve specific lysine ε-amino groups that surround the exposed heme edge of cytochrome *c* [11–13]. In the light of this background, we wished to investigate the possibility that a cytochrome *c*-552-cytochrome *c* complex could act as the catalytically active species in the sulfide:cytochrome *c* oxidoreductase activity of *C. vinosum* cytochrome *c*-552. Furthermore, we wished to determine the chemical identity of the oxidized sulfur-containing product of this reaction.

Materials and Methods

C. vinosum was grown on the malate-containing medium described by Knaff and Buchanan [14]. Cytochrome *c*-552 was isolated from the bacteria by the method of Bartsch [7]. The resulting protein (in its oxidized form) had absorbance ratios (A_{280}/A_{410}) that varied from 0.56 to 0.58.

Horse heart cytochrome *c*, Type VI, was obtained from Sigma Chemical Co. and used without further purification. Trifluoroacetylated cytochrome *c* was prepared by the method of Fanger and Harbury [15] using *S*-ethyl trifluorothioacetate obtained from Pierce Chemical Co. (Rockford, IL).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed on cytochrome *c*-552 by a slight modification of the method of Weber and Obson [16]. Bio-Phore 7.5% prepoured gels were used after overnight equilibration with Bio-Phore SDS Buffer obtained from Bio-Rad Laboratories. Protein molecular weight standards were obtained from Sigma Chemical Co. and Bio-Rad Laboratories.

Analysis for the presence of sulfate, sulfite and thiosulfate as oxidized sulfur products from the reaction of sulfide with cytochrome *c* in the presence of cytochrome *c*-552 was performed via ion chromatography after treatment of the reaction mixtures to remove protein. Cytochrome *c* was reduced in the presence of sulfide and catalytic amounts of cytochrome *c*-552 at ambient temperature in 1 mM Tricine-KOH buffer (pH 8.5). the reaction mixture was acidified to pH 4.5 by the additon of 0.1 M HCl and a 1000-fold excess of CM-Sephadex C-50-120 was added to remove the proteins from solution. After filtering the resin from solution, the solution was lyophilized and the residue dissolved in 2 ml of buffer. The dissolved residue was passed through a Diaflow UM2 ultrafiltration membrane and the filtrate analyzed by ion chromatography. An identical procedure was carried out with a sample lacking cytochrome *c*-552 as a blank control. Also, samples of standard solutions of each of the three ions were treated in the same manner to insure that none of the ions were lost during manipulation. Essentially complete recovery was obtained for all three anions. Ion chromatography of the solutions was performed on a Model 10 Dionex Ion Chromatograph using an anion-separator column as described by Holcombe et al. [17].

In assaying for elemental sulfur, cytochrome *c* was reduced by sulfide with cytochrome *c*-552 in a 5 mM Tricine-KOH buffer (pH 8.5) at ambient temperature. The elemental sulfur was extracted from the reaction mixture by shaking with carbon tetrachloride overnight. The carbon tetrachloride extract was washed twice with doubly distilled water and then evaporated. The dried residue was redissolved in a known carbon tetrachloride volume, and its aborbance at 280 nm was compared to a standard curve to determine sulfur concentration. A blank lacking cytochrome *c*-552 was run for comparison and an appropriate standard of elemental sulfur was run to determine the percentage of recovery.

A calibrated Sephacryl S-200 column (2.5 × 99

cm) was used to determine the molecular weight of cytochrome *c*-552 and its complex with cytochrome *c*. Elutions were carried out using the column preequilibrated at low ionic strength (30 mM Tris-HCl, pH 8), intermediate ionic strength (40 mM KCl, 5 mM Tris-HCl, pH 8) or high ionic strength (500 mM KCl, 30 mM Tris-HCl, pH 8) depending on the experimental requirements. Protein molecular weight standards were obtained from Pharmacia and Sigma Chemical Co.

The sulfide:cytochrome *c* oxidoreductase activity of cytochrome *c*-552 and all absorption spectra were measured on an Aminco DW-2a spectrophotometer. The activity was followed by monitoring the absorbance change at 550 minus 504 nm, caused by the reduction of cytochrome *c*, as a function of time. The reaction was performed in a single 1 ml cuvette and was initiated by the addition of small volumes of an Na_2S solution. The ionic strength of the reaction was varied by the additon of small aliquots of KCl, NaCl, $MgCl_2$, or Na_2SO_4 solutions.

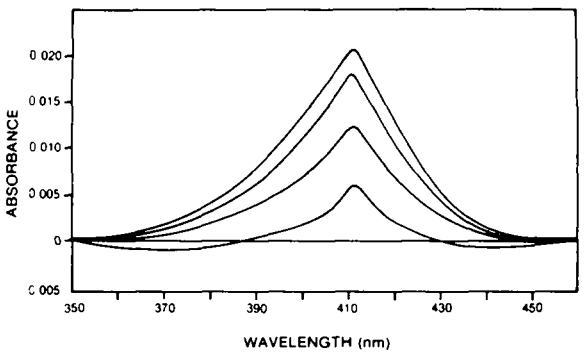

Fig. 1. Absorbance changes of cytochrome *c*-552 as a function of ionic strength. 1 ml samples of 5.7 μM cytochrome *c*-552 in 5 mM Tris-HCl (pH 8.0) were placed in both the sample and reference cuvette and the flat baseline at zero absorbance recorded. Aliquots of 1 M KCl were added to the sample cuvette to adjust the ionic strength and equal aliquots of water were added to the reference cuvette. The bottom difference spectrum was obtained with a final KCl concentration of 5.0 mM. The other three difference spectra, in order of increasing absorbance at 411 nm, were obtained after additions of KCl to give final concentrations of 14.8, 24.4 and 38.5 mM, respectively. Further additions of KCl produced no further changes in absorbance.

Results

The cytochrome *c*-552 purified for this investigation was subjected to SDS-polyacrylamide gel electrophoresis to insure the molecular weight and subunit structure corresponded to that previously reported [5,6]. Two Coomassie blue-staining bands were found with molecular weights of 46 600 and 21 300, giving a total molecular weight of 67 900. This is in good agreement with the values reported by Bartsch [5] and by Fukumori and Yamanaka [6]. When cytochrome *c*-552 was passed over a Sephacryl S-200 column equilibrated at either intermediate or high ionic strength, it moved with an apparent molecular weight of 64 000 ± 7000. The sample recovery rate was always at least 85%. However, when the column was reequilibrated with low ionic strength buffer, cytochrome *c*-552 migrated as a single, symmetrical band with an apparent molecular weight of 101 000 ± 7000. Similar results were obtained using a Sephadex G-100 column. Apparent anomalies in molecular weight determinations of this cytochrome on molecular-exclusion columns have been reported previously [18]. The apparent molecular weight at low ionic strength is not exactly twice that observed at higher ionic strengths, as would be expected if dimerization were occurring. Thus, it appeared possible that some conformational change affecting the hydrodynamic properties of the cytochrome occurred at low ionic strength. Fig. 1 shows that as the ionic strength is varied over the same range in which the apparent molecular weight of cytochrome *c*-552 decreases, the extinction coefficient of the heme Soret band of the oxidized cytochrome increases. This increase in the absorbance at 411 nm reached an end point when KCl had been added to a final concentration of 38.5 mM. When cytochrome *c*-552 was applied to a Sephacryl S-200 column at this ionic strength, the cytochrome eluted with an apparent molecular weight of 62000 ± 7000, the same value observed at all higher ionic strengths. Thus, there appears to be a good correlation between the ionic-strength-dependent spectral changes (possibly reflecting a conformation change in cytochrome *c*-552) and the changes in apparent molecular weight.

Because the ionic strength inside a *C. vinosum* cell is almost certainly higher than 40 mM [19], it was decided to conduct the majority of subsequent experiments at intermediate and high ionic

strengths. The questions of greatest interest were whether cytochrome *c*-552 forms a complex with cytochrome *c* and whether this complex is essential to the ability of the flavocytochrome to catalyze the oxidation of sulfide. Attempts were made to detect a complex between cytochrome *c*-552 and cytochrome *c* by passing mixtures of the two proteins over Sephacryl S-200 or Sephadex G-100 columns. Mixtures of cytochrome *c* and cytochrome *c*-522 in 1:1 or 2:1 mole ratios, when applied to the colums at intermediate ionic strength, elute as a single heme-containing band. Fig. 2A shows a typical experiment with 2:1 mole ratio mixture of cytochromes *c* and *c*-552. As can be seen, there is no detectable heme-containing protein appearing in fractions centered around fraction 78 where free cytochrome *c* would be expected to elute. At higher mole ratios of cytochrome *c* to *c*-552, free cytochrome *c* was detected. The behavior illustrated in Fig. 2A strongly suggests that cytochromes *c* and *c*-522 form a complex of high affinity at intermediate ionic strength. It should be mentioned that similar behavior is observed with the low ionic strength form of cytochrome *c*-552. At low ionic strengths, cytochrome *c*/*c*-552 mixtures elute from a Sephacryl S-200 column as a single band with an apparent molecular weight of 122000 ± 7000, with no free cytochrome *c* being observed (data not shown). The behavior at low and intermediate ionic strengths is

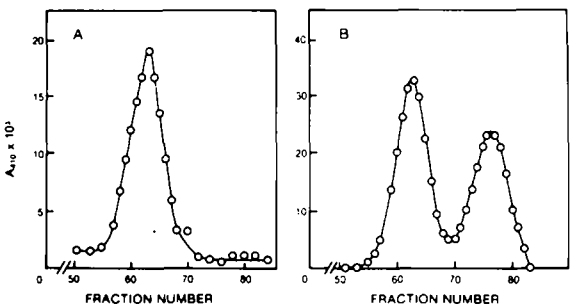

Fig. 2. The effect of ionic strength on the complex between cytochrome *c* and cytochrome *c*-552. (A) A 1 ml mixture of cytochromes *c* and *c*-552 (2:1 mole ratio) at an ionic strength of 43 mM was eluted from a Sephacryl S-200 column as described in Materials and Methods. The original cytochrome *c*-552 concentration was 4.4 μM. (B) A 1 ml mixture of cytochromes *c* and *c*-552 (2:1 mole ratio) at an ionic strength of 515 mM eluted from a Sephacryl S-200 column. The original cytochrome *c*-552 concentration was 11 μM.

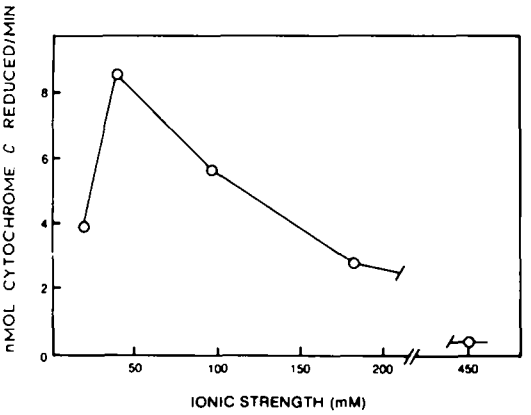

Fig. 3. The effect of an ionic strength on the sulfide:cytochrome *c* oxidoreductase activity of cytochrome *c*-552. The reaction mixtures contained 17.1 μM cytochrome *c*, 10 μM Na_2S and 40 nM cytochrome *c*-552. Aliquots of 2 M KCl were used to adjust the ionic strength. Other reaction conditions were as described in Materials and Methods. The rates of cytochrome *c* reduction observed in the absence of cytochrome *c*-552 were always less than 10% of those observed with the complete system.

in sharp contrast to that observed at high ionic strength. As can be seen in Fig. 2B, when a 2:1 mixture of cytochromes *c* and *c*-522 at high ionic strength was eluted from a Sephacryl S-200 column, the two proteins migrated independently. Essentially all the cytochrome *c* present in the original mixture was recovered in the free cytochrome *c* fraction under these conditions.

Having established the existence of a cytochrome *c*-*c*-552 complex, it was of interest to investigate the role of this complex in the sulfide:cytochrome *c* oxidoreductase activity of cytochrome *c*-552. As shown in Fig. 3, the rate of cytochrome *c* reduction (or sulfide oxidation) catalyzed by cytochrome *c*-552 increases to a maximum at ionic strengths in the 35–40 mM range and then declines dramatically as the ionic strength is increased. The results illustrated in Fig. 3 were obtained using KCl to adjust the ionic strength but essentially identical results were obtained using NaCl, $CaCl_2$ or Na_2SO_4, indicating that an ionic strength rather than specific ion effect is involved. Also, similar ionic strength dependencies of activity were observed over a wide range of substrate (sulfide and cytochrome *c*) concentrations. Since maximal rates of cytochrome *c* reduction were

obtained at an ionic strength where the cytochrome *c*-*c*-552 complex is known to be of high affinity (40 mM) and the rate of the reaction is essentially zero at an ionic strength (450 mM) where the complex is fully dissociated, it appears likely that the complex is the catalytically active species. Further evidence comes from the observation (data not shown) that at an ionic strength of 250 mM (where activity is typically less than 20% of that observed at 40 mM ionic strength) no complex between cytochromes *c*-552 and *c* could be observed on a Sephacryl S-200 column.

The correlation between complex formation and activity as a function of ionic strength not only suggests that the complex is the catalytically active species but that electrostatic forces predominate in stabilizing the complex. To test this hypothesis further, we eliminated the positive charges on cytochrome *c* resulting from lysine residues by trifluoroacetylating these residues. Trifluoracetylated cytochrome *c* is not reduced by sulfide in the presence of cytochrome *c*-552. Furthermore, when a 2:1 mixture of trifluoroacetylated cytochrome *c* and native cytochrome *c*-552 was applied to a Sephacryl s-200 column at intermediate ionic strength, the proteins migrated independently. It can thus be concluded that no complex forms between cytochrome *c*-552 and trifluoroacetylated cytochrome *c*.

With the documentation of the importance of complex formation for the oxidation of sulfide established, we turned to an identification of the oxidized sulfur-containing product of the reaction. Although it had previously been suggested [18] that elemental sulfur might be the product of the reaction, it was neccessary to obtain direct evidence as to the chemical identity of the oxidized sulfur-containing product. Reaction mixtures were assayed for four possible sulfur-containing products as described in Materials and Methods. As can be seen in Table I, no sulfate, sulfite or thiosulfate was detected. However, elemental sulfur was detected in an essentially stoichiometric amount. These results are consistent with elemental sulfur being the product of sulfide oxidation but the possibility does exist that other products are actually formed and decompose to S^0 during extraction.

TABLE I

IDENTIFICATION OF THE PRODUCT OF SULFIDE OXIDATION IN THE SULFIDE:CYTOCHROME *c* OXIDOREDUCTASE REACTION

The reaction mixtures analyzed for SO_3^{2-}, $S_2O_3^{2-}$ and SO_4^{2-} contained 16.5 μM cytochrome *c*, 10 μM Na_2S and 40 nM cytochrome *c*-552 in a volume of 40 ml. The reaction mixture analyzed for elemental sulfur contained 17.2 μM cytochrome *c*, 10 μM Na_2S and 40 nM cytochrome *c*-552 in a volume of 100 ml. Other conditons were as described in Materials and Methods. The amount of cytochrome *c* reduced was measured, as described in Material and Methods, for each sample and corrected for the nonspecific rate observed in the absence of cytochrome *c*-552. This correction never exceeded 10% of the rate observed for the complete system.

Possible product	μmol cytochrome c^{3+} reduced	μmol product predicted	μmol product observed
SO_3^{2-}	0.660	0.110	<0.008
$S_2O_3^{2-}$	0.660	0.165	<0.008
SO_4^{2-}	0.660	0.080	<0.008
S^0	1.72	0.86	0.74

Discussion

C. vinosum cytochrome *c*-552 appears to exist in two different conformational forms, one of which predominates at low ($I \leq 15$ mM) ionic strength and one of which predominates at higher ionic strengths. Both forms of this flavocytochrome appear to be able to form complexes with horse heart cytochrome *c*. Considering the internal concentrations of Na^+ and K^+ found in *C. vinosum* cells [19], it is unlikely that the low ionic strength form of cytochrome *c*-552 plays a significant role in vivo. Furthermore, as can be seen in Fig. 3, the activity of this form of the flavocytochrome in transferring electrons from sulfide to cytochrome *c* is considerably lower than that of the form that predominates at higher ionic strengths. However, work is continuing in an attempt to characterize further the nature of the low ionic strength form of cytochrome *c*-552.

The failure to detect any free cytochrome *c* in 1:1 or 2:1 cytochrome *c*/cytochrome *c*-552 mixtures at low or intermediate ionic strength suggests that the complex is one of high affinity, since no dissociation of the cytochromes occurs during

migration through the molecular-exclusion columns. These results also suggest that the stoichiometry of the complex is at least 2 cytochrome *c*: 1 cytochrome *c*-552. The observation that free cytochrome *c* is observed if the cytochrome *c*: *c*-552 mole ratio exceeds 2 suggests that there are not more than two high-affinity sites for cytochrome *c* on cytochrome *c*-552. An attempt was made to detect spectral changes accompanying the formation of the complex so that absorbance spectra could be exploited to establish the stoichiometry of the complex. No such spectral changes were observed but experiments using other techniques to determine the stoichiometry are currently underway.

A 2:1 cytochrome *c*-cytochrome *c*-552 complex would be consistent with our demonstration that sulfide is oxidized to elemental sulfur in the reaction catalyzed by cytochrome *c*-552. The two electrons donated by sulfide could be accepted by the FAD group of the cytochrome *c*-552 and then transferred, one each, to the two heme *c* groups of cytochrome *c*-552. Each ferroheme of cytochrome *c*-552 would then reduce one ferricytochrome *c*. Such a mechanism also is consistent with the data on intramolecular electron movements in cytochrome *c*-552 obtained by Cusanovich and Tollin [20] and by Kitagawa et al. [21]. This mechanism is summarized in Fig. 4, with the *C. vinosum* cytochrome *c*-551 replacing the horse heart cytochrome *c* used in our model systems. In view of the possibility that *C. vinosum* cytochrome *c*-551 may be structurally related to mitochondrial cytochrome *c*, it is extremely interesting that electrostatic complexes involving lysine residues may play as important a role in an anaerobic, photosynthetic prokaryote as they do in aerobic respiring eukaryotes.

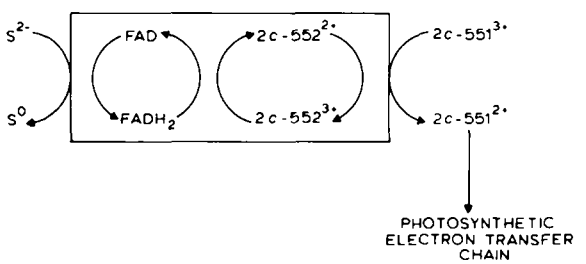

Fig. 4. Proposed pathway for electron transfer from sulfide to cytochrome *c*-551 catalyzed by cytochrome *c*-552 in *C. vinosum*.

Acknowledgements

This work was supported by grants (to D.B.K.) from the Robert A. Welch Foundation (D-710), the National Science Foundation (PCM-8109635) and the Texas Tech Institute of College Research. The authors would like to thank Professor Richard Bartsch for the use of his ion chromatography facilities, Professor Robert Holwerda and Professor Purnendu Dasgupta for their advice on the elemental sulfur determinations and Professor Robert Shaw for his insights into the nature of cytochrome complexes.

References

1 Van Niel, C.B. (1936) Arch. Mikrobiol. 7, 323–358
2 Schmidt, G.L. and Kamen, M.D. (1980) Arch. Mikrobiol. 73, 1–18
3 Smith, A.J. and Lascelles, J. (1966) J. Gen. Mikrobiol. 42, 357–370
4 Pfenning, N. (1975) Plant Soil 43, 1–16
5 Bartsch, R.G., Meyer, T.E. and Robinson, A.B. (1968) in Structure and Function of Cytochromes (Okunuki, K., Kamen, M.D. and Sekuzu, I., eds), pp. 443–451, University of Tokyo Press, Tokyo
6 Fukumori, Y. and Yamanaka, T. (1979) J. Biochem. 85, 1405–1414
7 Bartsch, R.G. (1971) Methods Enzymol. 34, 334–363
8 Van Grondelle, R., Duysens, L.N.M., Van der Wel, J.A. and Van der Wal, H.N. (1977) Biochim. Biophys. Acta 461, 188–201
9 Knaff, D.B., Whetstone, R. and Carr, J.W. (1980) Biochim. Biophys. Acta 590, 50–58
10 Rodkey, F.L. and Ball, E.G. (1950) J. Biol. Chem. 182, 17–28
11 Webb, M., Stronehuerner, J. and Millet, F. (1980) Biochim. Biophys. Acta 593, 290–298
12 Rieder, R. and Bosshard, H.R. (1980) J. Biol. Chem. 255, 4732–4739
13 Speck, S.H., Koppenol, W.H., Dethmers, J.K. Osherhoff, N., Margoliash, E. and Rajagopalan, K.U. (1981) J. Biol. Chem. 256, 7394–7400
14 Knaff, D.B. and Buchanan, B.B. (1975) Biochim. Biophys. Acta 376, 549–560
15 Fanger, M.W. and Harbury, H.A. (1965) Biochemistry 4, 2541–2545
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
17 Holcombe, L.T., Jones, B.F., Ellsworth, E.E. and Meserele, F.B. (1979) in Ion Chromatographic Analysis of Environmental Pollutants (Mulik, J.D. and Sawicki, E., eds), vol. 2,

pp. 401–412, Ann Arbor Science Publisher, Inc., Ann Arbor
18 Yamanaka, T. and Fukumari, Y. (1980) in Flavins and Flavoproteins (Yagi, K. and Yamano, T., eds), pp. 631–639, Japan Scientific Society Press, Tokyo
19 Davidson, V.L. and Knaff, D.B., Arch. Biochem Biophys., 213, 358–362
20 Cusanovich, M.A. and Tollin, G. (1980) Biochemistry 19, 334–347
21 Kitagawa, T., Fukumari, Y. and Yamanaka, T. (1980) Biochemistry 19, 5721–5729