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Complex formation between *Chlorobium limicola* f. *thiosulfatophilum* *c*-type cytochromes

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It has been possible to demonstrate, using affinity chromatography, that *Chlorobium* flavocytochrome *c*-553 forms an electrostatically stabilized complex with *Chlorobium* cytochrome *c*-555. The binding site for cytochrome *c*-555 appears to be located on the heme-containing subunit of flavocytochrome *c*-553. This complex appears to be involved in the flavocytochrome *c*-553-catalyzed transfer of electrons from sulfide to cytochrome *c*-555. Complex formation has also been demonstrated between *Chlorobium* cytochromes *c*-555 and *c*-551, two components involved in the oxidation of thiosulfate by this green sulfur bacterium. Affinity chromatography data also suggest the possibility that the cytochrome binding sites on the *Chlorobium* flavocytochrome *c*-553 and on flavocytochrome *c*-552 from the purple sulfur bacterium *Chromatium vinosum* may be similar.

Introduction

Among the cytochromes found in the photosynthetic green sulfur bacterium *Chlorobium limicola* f. *thiosulfatophilum* (hereafter, *Chlorobium*) are three soluble *c*-type cytochromes that appear to be involved in the oxidation of sulfur-containing electron donors by this bacterium. Flavocytochrome *c*-553 [1], which consists of a 47 kDa subunit containing a single covalently bound FAD and an 11 kDa subunit containing a single heme *c* [2–6], has been demonstrated to catalyze the oxidation of sulfide [7–9]. Although a number of *c*-type cytochromes can function as electron acceptors for sulfide oxidation catalyzed by flavocytochrome *c*-553 in vitro, the in vivo acceptor appears to be another soluble *c*-type cytochrome, cytochrome *c*-555 [7–9]. Cytochrome *c*-555, which has been found in all photosynthetic green sulfur bacteria studied to date [10], has been shown by X-ray crystallography [11,12] to possess structural similarities to mitochondrial cytochrome *c* and cytochrome c_2 from the photosynthetic purple non-sulfur bacterium *Rhodospirillum rubrum*. Cytochrome *c*-555, in addition to its likely role as an electron acceptor during the oxidation of sulfide, can also function as an acceptor of electrons from thiosulfate. This oxidation of thiosulfate in *Chlorobium* (and other thiosulfate-oxidizing photosynthetic green sulfur bacteria) involves as the initial electron acceptor a third soluble cytochrome, cytochrome *c*-551 [8,10,13,14].

The photosynthetic purple sulfur bacterium *Chromatium vinosum* contains a soluble flavocytochrome *c*-552 [15], similar in many ways to the *Chlorobium* flavocytochrome *c*-553 [2,4,5,9,16]. It has been demonstrated that the sulfide/cytochrome *c* oxidoreductase activity of the *C. vino-*

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sum flavocytochrome *c*-552 [16,17] involves an electrostatic complex with the high-potential *c*-type cytochromes that can serve as acceptors of electrons during the oxidation of sulfide to elemental sulfur [17]. The existence of a complex between *C. vinosum* flavocytochrome *c*-552 and equine cytochrome *c*, stabilized by electrostatic forces, has been confirmed by gel filtration [18], affinity chromatography [19], chemical modification [20] and cross-linking studies [21]. In view of the similarities between the *C. vinosum* and *Chlorobium* flavocytochromes *c*, it is of interest to determine whether electrostatic complex formation between *Chlorobium* flavocytochrome *c*-553 and cytochrome *c*-555 occurs. We have obtained evidence for such a complex and for its role in sulfide oxidation and, furthermore, have demonstrated additional similarities between the two flavocytochromes. Moreover, evidence has been obtained for complex formation between *Chlorobium* cytochromes *c*-555 and *c*-551.

Materials and Methods

C. limicola f. *thiosulfatophilum* strain Tassajara soluble cytochromes *c*-555, *c*-551 and flavocytochrome *c*-553 were purified according to the method of Meyer et al. [1]. Cytochrome c_2 from *Rhodospirillum rubrum* S1 and the flavocytochrome *c*-552 from *C. vinosum* strain D were purified as described by Bartsch [22]. The heme subunit of the *C. vinosum* flavocytochrome *c*-552 was prepared as described by Vorkink [23] and Brown [24]. The heme subunit of the *Chlorobium* flavocytochrome *c*-553 was prepared as described by Yamanaka et al. [5]. The heme subunit preparations had a A_{280}/A_{410} ratio of 0.20 or less, lacked any absorbance features attributable to FAD and exhibited single Coomassie blue-staining bands after polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) with M_r 21 000 and 11 000 for the *C. vinosum* and *Chlorobium* proteins, respectively. All proteins were subjected to polyacrylamide gel electrophoresis in the presence of SDS and high performance liquid chromatography (HPLC) analysis to ensure purity. Gel electrophoresis was performed on 14% slab gels (1.5 mm thickness) prepared according to O'Farrell [25]. HPLC analysis was performed on a Waters Associates system using a Protein Pak 125 gel filtration column.

Equine cytochrome *c* (Type VI) and *Saccharomyces cerevisiae* cytochrome *c* were obtained from Sigma Chemical Co. The equine cytochrome *c* was purified further by ion-exchange chromatography on CM-Sephadex while the *S. cerevisiae* cytochrome *c* was used without further purification. Trifluoroacetylation of equine cytochrome *c* was accomplished by using the method of Fanger and Harbury [26].

Affigel-10 was obtained from Bio-Rad and cyanogen bromide-activated Sepharose 4B from Pharmacia. Proteins were coupled to the affinity matrix according to the procedures outlined by the manufacturers [27,28]. The equine cytochrome *c*-Sepharose 4B and the *C. vinosum* flavocytochrome *c*-552-Affigel-10 matrices were prepared as described previously [19]. The *Chlorobium* cytochrome *c*-555-Sepharose 4B matrix was prepared using 200 nmol of cytochrome *c*-555 and 1.01 g cyanogen bromide-activated Sepharose 4B.

Absorbance spectra (spectral resolution, 1.0 nm) were obtained using Aminco DW-2a and Perkin-Elmer Lambda 5 spectrophotometers. Sulfide/cytochrome *c* oxidoreductase activity was measured using the Aminco DW-2a spectrophotometer as previously described [17].

Results

Previous studies [17,18] using gel filtration have established that *C. vinosum* flavocytochrome *c*-552 and equine cytochrome *c* form a stable complex which elutes as a single band at low ionic strengths (up to 43 mM). In contrast, similar gel filtration studies with *Chlorobium* flavocytochrome *c*-553 detected no complex with either equine cytochrome *c* or *Chlorobium* cytochrome *c*-555 at ionic strengths as low as 10 mM [18]. However, the gel filtration, co-chromatography approach requires a fairly high association constant (at least $1 \cdot 10^5$ M^{-1}) for a complex to be detectable. For this reason, the binding of *Chlorobium* flavocytochrome *c*-553 to *Chlorobium* cytochrome *c*-555 and equine cytochrome *c* was reinvestigated using affinity chromatography. Fig. 1A shows that the *Chlorobium* flavocytochrome *c*-553 does form a complex with cytochrome *c*-555, as evidenced by

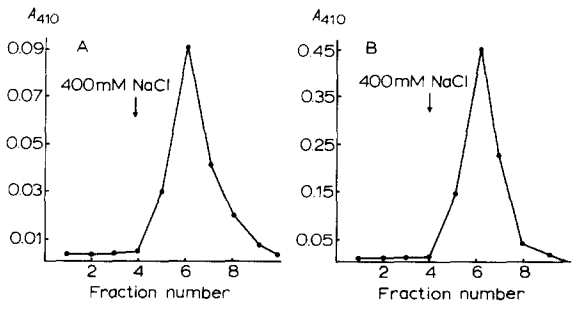

Fig. 1. (A) *Chlorobium* flavocytochrome *c*-553 binding to a cytochrome *c*-555-Sepharose 4B affinity column. 50 nmol of flavocytochrome *c*-553 in 15 mM Tris buffer (pH 8.0) were applied to the affinity column (bed volume, 3.5 ml) which had been equilibrated with the same buffer. The column was washed with the equilibration buffer and then the ionic strength of the eluting buffer was increased by NaCl addition where indicated. 1.7 ml fractions were collected. Total recovery of flavocytochrome *c*-553 was 91%. (B) *Chlorobium* flavocytochrome *c*-553 binding to an equine cytochrome *c*-Sepharose 4B affinity column. 100 nmol flavocytochrome *c*-553 in 15 mM Tris buffer (pH 8.0) were applied to the affinity column (bed volume, 5.3 ml) which had been pre-equilibrated with the same buffer. The column was washed with the equilibration buffer and then the ionic strength of the eluting buffer was increased by NaCl addition where indicated. 1.4 ml fractions were collected. Total recovery of flavocytochrome *c*-553 was 92%.

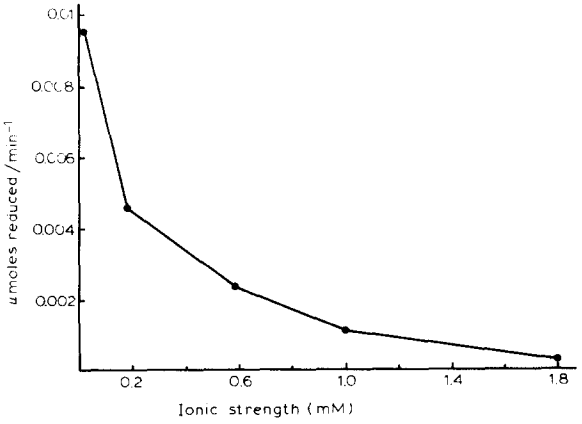

Fig. 2. The effect of ionic strength on the sulfide/cytochrome *c*-555 oxidoreductase activity of *Chlorobium* flavocytochrome *c*-553. The reaction mixtures contained 10 μM cytochromes *c*-555/25 μM Na_2S/40 nM flavocytochrome *c*-553 in 5 mM Tris buffer (pH 8.0). Aliquots of 2 M KCl were used to adjust the ionic strength.

its ability to bind to a cytochrome *c*-555 affinity column. Evidence for the electrostatic nature of the complex comes from the observation that flavocytochrome *c*-553 can be eluted from the cytochrome *c*-555-Sepharose 4B affinity column by increasing the ionic strength. In control experiments (data not shown), it was found that the *Chlorobium* flavocytochrome *c*-553 did not bind to a Sepharose 4B column that contained no cytochrome *c*-555, but instead had the cyanogen bromide groups reacted with glycine. This demonstrates that the flavocytochrome *c*-553 binds to the affinity ligand, cytochrome *c*-555, rather than non-specifically to the Sepharose 4B matrix. As equine cytochrome *c* can serve as an electron acceptor in the in vitro oxidation of sulfide catalyzed by *Chlorobium* flavocytochrome *c*-553 (see below), it was of interest to utilize affinity chromatography to investigate whether complex formation occurred between equine cytochrome *c* and flavocytochrome *c*-553. Fig. 1B shows that the flavocytochrome *c*-553 does indeed bind to a cytochrome *c*-Sepharose 4B affinity column. As was the case for the cytochrome *c*-555 affinity column, increasing the ionic strength eluted the flavocytochrome *c*-553 from the cytochrome *c*-Sepharose 4B affinity column.

Evidence consistent with the involvement of an electrostatically stabilized complex between the *Chlorobium* flavocytochrome *c*-553 and cytochrome *c*-555 in the oxidation of sulfide comes from the observation (Fig. 2) that the rate of the reaction decreases dramatically with increasing ionic strength with an optimum below 10 mM ionic strength. Thus the reaction becomes unfavorable under conditions where an electrostatic complex would tend to dissociate. Similar reaction rate versus ionic strength profiles were observed for the flavocytochrome *c*-553-catalyzed electron transfer from sulfide to equine and *S. cerevisiae* cytochromes *c* and to *R. rubrum* cytochrome c_2 (data not shown). While equine cytochrome *c* can function as an alternative in vitro electron acceptor for the native *Chlorobium* cytochrome *c*-555, its structure appears to be sufficiently different from that of cytochrome *c*-555 to produce large changes in the kinetic parameters of the sulfide/cytochrome *c* oxidoreductase reaction catalyzed by flavocytochrome *c*-553. Thus, K_m values of 14 and 600 μM (at 2.5 mM ionic strength) were

determined in this study for cytochrome *c*-555 and equine cytochrome *c*, respectively. The V_{max} for equine cytochrome *c* was 13-fold greater than that for the native cytochrome *c*-555 (7.5 mmol/min per μmol cytochrome *c*-553 for equine cytochrome *c* vs. 0.56 mmol/min per μmol cytochrome *c*-553 for cytochrome *c*-555).

A large amount of data supports the hypothesis that positive charges from specific lysine residues on mitochondrial cytochrome *c* are involved in stabilizing the electrostatic complexes cytochrome *c* forms with a number of its reaction partners [12,29]. It has recently been shown that these same lysine residues are involved in forming a complex between cytochrome *c* (used as an in vitro analog for the native *C. vinosum* cytochrome *c*-550 [19]) and the *C. vinosum* flavocytochrome *c*-552 [20]. Evidence that these lysine residues may also be involved in the ability of cytochrome *c* to function as an acceptor of electrons from sulfide in the reaction catalyzed by the *Chlorobium* flavocytochrome *c*-553 was provided by the observation that equine cytochrome *c* in which all of the lysine ε-amino groups were trifluoroacetylated [27] to remove their positive charges did not function as an electron acceptor in the in vitro assay system (data not shown).

In view of the considerable catalytic and structural similarities between the *C. vinosum* flavocytochrome *c*-552 and *Chlorobium* flavocytochrome *c*-553, and of our finding in this study that cytochrome *c*-555 can serve as an electron acceptor in the *C. vinosum* flavocytochrome *c*-552 catalyzed oxidation of sulfide, it was of interest to investigate whether heterogeneous complexes between cytochromes from the two bacteria could be formed. Such complexes have in fact been detected by affinity chromatography. *Chlorobium* cytochrome *c*-555 does bind to *C. vinosum* flavocytochrome *c*-552 that has been covalently attached [19] to an Affigel-10 matrix. Further evidence for the hypothesis that the cytochrome complexing sites on the two flavocytochromes are similar comes from the observation that *C. vinosum* flavocytochrome *c*-552 binds to a *Chlorobium* cytochrome *c*-555-Sepharose 4B affinity column. These heterogeneous complexes appear to be electrostatic in nature, since increasing ionic strength elutes both proteins from the respective affinity

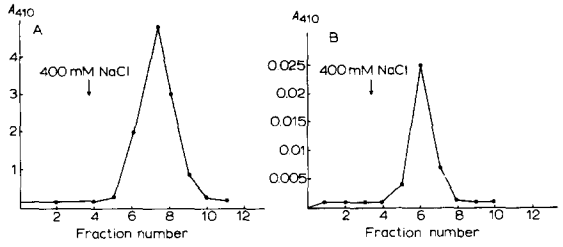

Fig. 3. (A) *Chlorobium* flavocytochrome *c*-553 heme subunit binding to the cytochrome *c*-555-Sepharose 4B affinity column. 15 nmol heme subunit in 15 mM Tris buffer (pH 8.0) were applied to the column which had been equilibrated with the same buffer. After washing with the equilibration buffer, the ionic strength of the elution buffer was raised by the addition of NaCl to a final value of 400 mM. 1.0 ml fractions were collected. Total recovery of the heme subunit was 88%. (B) *C. vinosum* flavocytochrome *c*-552 heme subunit binding to the cytochrome *c*-555-Sepharose 4B affinity column. 11 nmol heme subunit in 20 mM Tris buffer (pH 8.0) were applied to the column which had been equilibrated with the same buffer. After washing with the equilibration buffer, the ionic strength of the elution buffer was raised by the addition of NaCl to a final concentration of 400 mM. 1.5 ml fractions were collected. Total recovery of the heme subunit was 94%

columns. As is the case for the *C. vinosum* flavocytochrome *c*-552 complex with cytochrome *c* [19], the heme subunits of the flavocytochromes appears to supply the major site for cytochrome *c*-555 binding. Fig. 3A shows that the heme subunit of the *Chlorobium* flavocytochrome *c*-553 binds to the cytochrome *c*-555-Sepharose 4B affinity column and Fig. 3B shows the binding of the *C. vinosum* flavocytochrome *c*-552 heme subunit to the cytochrome *c*-555-Sepharose 4B affinity column.

As discussed in the Introduction, a third soluble *Chlorobium* cytochrome, cytochrome *c*-551 is thought to interact with cytochrome *c*-555 in the pathway of thiosulfate oxidation, but not to be involved in the flavocytochrome *c*-553-catalyzed oxidation of slufide [8,10]. As would be expected from this hypothesis, we could detect no binding of cytochrome *c*-551 to the *C. vinosum* flavocytochrome *c*-552-Affigel 10 affinity column even at ionic strengths as low as 2.5 mM. This absence of binding provides some measure of confidence in the specificity of the affinity chromatography techniques used in this study. However, as can be

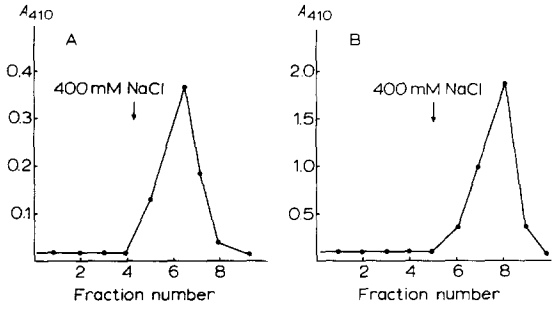

Fig. 4. (A) *Chlorobium* cytochrome *c*-551 binding to a cytochrome *c*-555-Sepharose 4B affinity column. 40 nmol of cytochrome *c*-551 in 20 mM Tris buffer (pH 8.0) was applied to the affinity column. Elution conditions were as in Fig. 3B. Total recovery of cytochrome *c*-551 was 99%. (B) *Chlorobium* cytochrome *c*-551 binding to an equine cytochrome *c*-Sepharose 4B affinity column. 38 nmol of cytochrome *c*-551 was applied to the column. Conditions were as in Fig. 1B except that 2.0 ml fractions were collected. Total recovery of cytochrome *c*-551 was 99%.

seen in Fig. 4A, cytochrome *c*-551 does bind to the cytochrome *c*-555-Sepharose 4B affinity column. Complex formation between the *Chlorobium* cytochromes *c*-551 and *c*-555 is consistent with their proposed roles [8] in thiosulfate oxidation by this photosynthetic green sulfur bacterium. In view of the existence of some structural similarities between cytochromes *c*-555 and mitochondrial cytochrome *c* [11,12], it was of interest to determine whether *Chlorobium* cytochrome *c*-551 would also bind to a cytochrome *c*-containing affinity column. Fig. 4B shows that cytochrome *c*-551 does bind to an equine cytochrome *c*-Sepharose 4B affinity column and, as is the case with the cytochrome *c*-555 affinity column, the cytochrome *c*-551 can be eluted by increasing the ionic strength.

Discussion

The data presented above support the hypothesis that *Chlorobium* flavocytochrome *c*-553 forms an electrostatically stabilized complex with cytochrome *c*-555 and that this complex functions in the sulfide/cytochrome c-555 oxidoreductase reaction catalyzed by the flavocytochrome. As electrostatic complex formation between the *C. vinosum* flavocytochrome *c*-552 and those cytochrome *c* that can function as electron acceptors from sulfide in the flavocytochrome *c*-552-catalyzed reaction had previously been demonstrated [17–21], it appears that complex formation between catalyst and electron acceptor proteins may be a general aspect of flavocytochrome-catalyzed sulfide oxidation in both photosynthetic purple and green sulfur bacteria. The facts that *Chlorobium* cytochrome *c*-555 can serve as an electron acceptor in the *C. vinosum* flavocytochrome *c*-552-catalyzed oxidation of sulfide, that *R. rubrum* cytochrome c_2 and equine and yeast cytochromes *c* can serve as electron acceptors in both systems and that the *C. vinosum* flavocytochrome *c*-552 can bind to a *Chlorobium* cytochrome *c*-555-Sepharose 4B affinity column further support the idea of the basic similarities of the two systems. In so far as the ability of equine cytochrome *c* to serve as an acceptor in the *Chlorobium* flavocytochrome *c*-553-catalyzed reaction is concerned, the unfavorable K_m for equine cytochrome *c* (600 μM) compared to that for the native cytochrome *c*-555 (14 μM) can likely be attributed to structural differences between the two cytochromes [11,12]. The somewhat surprising observation of a considerably larger V_{max} in this reaction for equine cytochrome *c* than for cytochrome *c*-555 may be due [31] to the larger thermodynamic driving force available with cytochrome *c* ($E_m = +260$ mV, Ref. 11) than with cytochrome *c*-555 ($E_m = +145$ mV, Ref. 32).

Cytochrome *c*-555 plays a central role in the sulfur metabolism of *Chlorobium*, in that it accepts electrons from both sulfide and thiosulfate [8]. In the case of thiosulfate oxidation, cytochrome *c*-555 is not the initial acceptor, but rather accepts electrons from cytochrome *c*-551, the substrate for the bacterium's, thiosulfate/cytochrome *c* oxidoreductase [8]. While cytochrome *c*-555 is a poor substrate for the oxidoreductase, it greatly stimulates the rate of cytochrome *c*-551 reduction by the enzyme, in addition to subsequently serving to re-oxidize reduced cytochrome *c*-551 [8]. The evidence presented above (see Fig. 4A) for complex formation between cytochromes *c*-551 and *c*-555 may provide an explanation for the observed stimulation. Either the complex between the two cytochromes may be the actual substrate for the thiosulfate/cytochrome *c* oxidoreductase or interactions between the two cytochromes on complex

formation produce a conformational change in cytochrome *c*-551 that makes it a better substrate for the enzyme.

Although the molecular details of the flavocytochrome *c*-cytochrome *c* interaction will have to await the results of further studies, the available data (this work and Refs. 18 and 33) are consistent with the flow of electrons from sulfide to the flavin moiety of flavocytochrome *c*, then to the heme moiety of the flavocytochrome *c* and finally to the heme group of the acceptor (cytochrome *c*-555 in *Chlorobium* and cytochrome *c*-550 in *C. vinosum*). Thus, the flavocytochromes *c* should provide a useful soluble model system for elucidating the effects of prosthetic group orientations and distances in biological electron-transfer reactions.

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References

1 Meyer, T.E., bartsch, R.G., Cusanovich, M.A. and Mathewson, J.H. (1968) Biochim. Biophys. Acta 153, 854–861
2 Yamanaka, T. and Kusai, A. (1976) in Flavins and Flavoproteins (Singer, T.P., ed.), pp. 292–301, Elsevier, Amsterdam
3 Yamanaka, T. (1976) J. Biochem. 79, 655–660
4 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
5 Yamanaka, T., Fukumori, Y. and Okunuki, K. (1979) Anal. Biochem. 95, 209–213
6 Kenney, W.C., McIntire, W. and Yamanaka, T. (1977) Biochim. BIophys. Acta 483, 467–474
7 Kusai, A. and Yamanaka, T. (1973) FEBS Lett. 34, 235–237
8 Kusai, A. and Yamanaka, T. (1973) Biochim. Biophys. Acta 325, 304–314
9 Yamanaka, T. and Fukumori, Y. (1980) in Flavins and Flavoproteins (Yagi, K. and Yamano, T., eds.), pp. 631–639, Japan Scientific Societies press, Tokyo
10 Trüper, H.G. and Fischer, U. (1982) Phil. Trans. R. Soc. Lond. B298, 529–542
11 Salemme, R.F. (1977) Annu. Rev. Biochem. 46, 299–329
12 Korszun, Z.R. and Salemme, F.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5244–5247
13 Kusai, A. and Yamanaka, T. (1973) Biochem. Biophys. Res. Comm. 51, 107–112
14 Fischer, U. (1984) Sulfur, Its Significance for Chemistry for the Geo-, Bio- and Cosmosphere and Technology (Müller, A. and Krebs, B., eds.), pp. 383–407, Elsevier, Amsterdam
15 Bartsch, R.G. and Kamen, M.D. (1960 J. Biol. Chem. 235, 825–821
16 Fukumori, Y. and Yamanaka, T. (1979) J. Biochem. 85, 1405–1414
17 Gray, G.O. and Knaff, D.B. (1982) Biochim. Biophys. Acta 680, 290–296
18 Meyer, T.E., Vorkink, W.P., Tollin, G. and Cusanovich, M.A. (1985) Arch. Biochem. Biophys. 236, 52–58
19 Davidson, M.W., Gray, G.O. and Knaff, D.B. (1985) FEBS Lett. 187, 155–159
20 Bosshard, H.R., Davidson, M.W., Knaff, D.B. and Millett, F. (1986) J. Biol. Chem. 261, 190–193
21 Vieira, B., Davidson, M., Knaff, D. and Millett, F. (1986) Biochim. Biophys. Acta 848, 131–136
22 Bartsch, R.G. (1971) Methods Enzymol. 23, 344–363
23 Vorkink, W.P. (1972) Ph.D. Thesis, University of Arizona
24 Brown, S.L. (1981) M.S. Thesis, University of Arizona
25 O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021
26 Fanger, M.W. and Harbury, H.A. (1965) Biochemistry 4, 2541–2545
27 Bio-Rad Laboratories Technical Bulletin (1982) 1085
28 Affinity Chromatography, Principles and Methods (1979) Pharmacia Fine CHemicals
29 Margoliash, E. and Bosshard, H.R. (1983) Trends Biochem. Sci. 8, 316–320
30 Knaff, D.B., Whetstone, R. and Carr, J.W. (1980) Biochim. Biophys. Acta 590, 50–58
31 Marcus, R.A. (168) J. Phys. Chem. 72 891–899
32 Gibson, J. (1961) Biochem. J. 79, 151–158
33 Cusanovich, M.A., Meyer, T.E. and Tollin, G. (1985) Biochemistry 24, 1281–1287