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Purification of hydrogenases by affinity chromatography on Procion Red–agarose

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The agarose-coupled triazine dye Procion Red HE-3B has been demonstrated to be applicable as an affinity gel for the purification of five diverse hydrogenases, namely the soluble, NAD-specific and the membrane-bound hydrogenase of *Alcaligenes eutrophus*, the membrane-bound hydrogenase of the N_2-fixing *Alcaligenes latus*, the reversible H_2-evolving and the unidirectional H_2-oxidizing hydrogenase of *Clostridium pasteurianum*. In the case of the soluble hydrogenase of *A. eutrophus*, chromatography on Procion Red–agarose even permitted the separation of inactive from active enzyme, thus yielding a 2–3-fold increase in specific activity. For the homogeneous enzyme preparation obtained after two column steps (Procion Red–agarose, DEAE-Sephacel), a specific activity of 121 μmol of H_2 oxidized/min per mg of protein was determined. Kinetic studies with free Procion Red provided evidence that the diverse hydrogenases are competitively inhibited by the dye, each with respect to the electron carrier (NAD, Methylene Blue, Methyl Viologen), indicating a specific interaction between Procion Red and the catalytic centres of the enzymes. For the highly purified preparations of the soluble and the membrane-bound hydrogenase of *A. eutrophus*, in 50 mM-potassium phosphate, pH 7.0, K_i values for Procion Red of 103 and 19 μM have been determined.

Since the first presentation of homogeneous or nearly homogeneous preparations of hydrogenase from *Clostridium pasteurianum* (Nakos & Mortenson, 1971; Chen & Mortenson, 1974) and *Desulfovibrio vulgaris* (Haschke & Campbell, 1971; LeGall *et al.*, 1971), hydrogenases have been isolated and purified from many diverse microorganisms (Schlegel & Schneider, 1978; Adams *et al.*, 1981). For most of the soluble as well as the solubilized membrane-bound enzymes well-established procedures of protein precipitation [$(NH_4)_2SO_4$ fractionation, heat treatment] and chromatography (gel filtration, ion-exchange) were applied. In recent years, several membrane-bound hydrogenases, e.g. the enzymes of *Azotobacter chroococcum* (Van der Werf & Yates, 1978), *Proteus mirabilis* (Schoenmaker *et al.*, 1979), *Escherichia coli* (Adams & Hall, 1979) and *Chromatium* (Van Heerikhuizen *et al.*, 1981) have also been chromatographed on the basis of hydrophobic interaction with octyl- or phenyl-Sepharose respectively. Although most of the purifications described in the literature were of relatively small scale, yielding only 1–2 mg of pure enzyme, the use of at least three to four column steps were necessary to achieve homogeneous protein preparations. Up to now, larger-scale purifications of hydrogenase have led to less-pure preparations and have required additional or alternative purification steps respectively (Van Heerikhuizen *et al.*, 1981; Schneider *et al.*, 1979). For the soluble, NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16 a preparative gel-electrophoresis procedure was elaborated (Schneider *et al.*, 1979). This method turned out to be highly effective in separating protein contaminants; however, the overall yield of enzyme activity was only 6%.

Affinity-chromatography methods have not yet been found to be applicable for the purification of hydrogenases. During the past few years, Cibacron Blue F3G-A and related dyes have been successfully used in affinity chromatography, particularly for the purification of enzymes interacting with nucleotides and other heterocyclic compounds (Dean & Watson, 1979; Lowe *et al.*, 1980). The availability of these triazine dyes prompted us to examine them for the capacity to bind hydrogenases.

In the present paper we describe improved purification procedures for the soluble NAD-specific as well as for the membrane-bound and respiratory-chain-linked hydrogenase of *Alcaligenes eutrophus* H16. Both procedures included affinity chromatography on Procion Red HE-3B–agarose as the essential step. We further demonstrate that hydro-

Abbreviation used: SDS, sodium dodecyl sulphate.

genases of other organisms, namely the aerobic N_2-fixing hydrogen bacterium *Alcaligenes latus* and the anaerobic N_2-fixing *Clostridium pasteurianum* bind to Procion Red HE-3B–agarose too, and that the reduction (NAD, Methylene Blue) and oxidation (Methyl Viologen) of electron carriers by the used enzymes is competitively inhibited by Procion Red.

Experimental

Chemicals

Procion Red HE-3B and related dyes coupled to agarose were obtained from Amicon G.m.b.H., Witten, Germany. The concentration of the coupled Procion Red was 2.5–4 mg/ml of swollen gel. About 5–10 mg of protein was bound to 1 ml of the gel. A sample of 95%-pure free Procion Red HE-3B was a gift from Dr. A. Steinbüchel (University of Göttingen, Göttingen, Germany) who originally obtained the dye from ICI (Frankfurt, Germany). DEAE-Sephacel was purchased from Pharmacia Fine Chemicals. The sources of all other chemicals used were as listed by Schneider & Schlegel (1976).

Growth of organisms

Alcaligenes eutrophus H16 (A.T.C.C. 17699, DSM 428) was grown heterotrophically in a mineral medium as described by Schlegel *et al.* (1961). The medium contained a mixture of 0.5% fructose and 0.2% glycerol as carbon sources (Friedrich *et al.*, 1981). *Alcaligenes latus* was grown autotrophically as described by Malik *et al.* (1981). Cells of *Clostridium pasteurianum* MR 505 (a granulose-negative mutant; Robson *et al.*, 1974), which were a gift from Dr. W. Andersch (University of Göttingen, Göttingen, Germany), were grown anaerobically with glucose as carbon source (Mackey & Morris, 1971).

Preparation of enzymes

Soluble hydrogenase (EC 1.18.3.1) from *A. eutrophus* H16 was purified up to the $(NH_4)_2SO_4$-fractionation step as described previously (Schneider & Schlegel, 1976; Schneider *et al.*, 1979). The $(NH_4)_2SO_4$ precipitate was dialysed against 50 mM-potassium phosphate buffer, pH 6.2, and then applied to a Procion Red HE-3B–agarose column (5 cm × 60 cm), pre-equilibrated with the same buffer. Protein which was not bound to the gel was removed by washing the column with the dialysis buffer. Hydrogenase was eluted with a linear 0–1 M-KCl gradient prepared in 50 mM-potassium phosphate buffer, pH 6.2. At a flow rate of 80 ml/h, fractions with a volume of 6 ml each were collected, and those fractions containing hydrogenase activity were combined, dialysed against 50 mM-potassium phosphate, pH 7.0, and loaded on to a column (2.5 cm × 40 cm) of DEAE-Sephacel. Protein elution was performed with a discontinuous (0.07, 0.09, 0.15, 0.25, 1 M) KCl gradient in 50 mM-potassium phosphate buffer, pH 7.0. The flow rate and fraction volume were 40 ml/h and 4 ml respectively. As analysed by polyacrylamide-gel electrophoresis (Schneider & Schlegel, 1976), homogeneous hydrogenase was present in fractions eluted with 0.09 M-KCl.

The membrane-bound hydrogenases of *A. eutrophus* and *A. latus* were solubilized (Schink & Schlegel, 1979), fractionated with 40–60% (w/v) $(NH_4)_2SO_4$ dialysed against 50 mM potassium phosphate buffer, pH 7.0, and subsequently applied to a column (2.5 cm × 40 cm) of Procion Red HE-3B–agarose. The elution conditions were as for the soluble hydrogenase.

A 3 g portion of frozen cells of *C. pasteurianum* were thawed in 10 ml of O_2-free 50 mM-potassium phosphate, pH 7.0, and were ruptured by passing them once through a French pressure cell (Aminco) at 147 MPa. The broken cells were collected anaerobically in a tube which was pre-gassed with H_2 and contained 1 mM-sodium dithionite. Particulate debris was removed by centrifugation at 10000 ***g*** for 20 min. A 2 ml portion of the clarified supernatant was loaded anaerobically on to a Procion Red HE-3B–agarose column (0.9 cm × 10 cm) which had been equilibrated with H_2-saturated 50 mM-potassium phosphate buffer, pH 6.2. Protein was first eluted with the equilibration buffer followed by the elution with a discontinuous KCl gradient (8 ml each of 0.1, 0.25, 0.5, 1 and 2 M-KCl in 50 mM-potassium buffer, pH 6.2). Fractions (8 ml each) were collected which were continuously gassed with O_2-free H_2 during elution.

Determination of hydrogenase activity

NAD and Methylene Blue reduction were determined as outlined by Schneider & Schlegel (1976) and Schink & Schlegel (1979) respectively. H_2 evolution was measured in a Warburg apparatus using reduced Methyl Viologen as electron donor. The Warburg flasks (total liquid volume 2.2 ml), which contained in their main compartment 10 mM-Methyl Viologen and 100 mM-potassium phosphate, pH 7.0, were shaken and flushed with O_2-free N_2 for 30 min. Sodium dithionite (15 mM), dissolved anaerobically in the test buffer, was added to the main compartment by a syringe after the gassing procedure. The reaction was started by tipping the enzyme extract from the side arm into the main compartment.

Protein determination

Protein was determined by the biuret method (Beisenherz *et al.*, 1953).

Results and discussion

Purification of soluble NAD-specific hydrogenase from Alcaligenes eutrophus

Five diverse agarose-bound triazine dyes, Cibacron Blue 3G-A (Blue A), Blue B, Procion Red HE-3B (Red A), Orange A and Green A [for details of the structures of the dyes, see Fulton (1980)] were examined as to their qualitative and quantitative capability for binding the hydrogenases of *Alcaligenes eutrophus* H16. Screening was performed in 2ml columns (1cm × 3.5cm) and with enzyme preparations obtained after $(NH_4)_2SO_4$ treatment (see the Experimental section).

The soluble NAD-specific hydrogenase was not bound at all by the immobilized dyes Blue B and Orange A and only weakly by Cibacron Blue. In the latter case, 20% of the hydrogenase activity was eluted merely by washing the column with the starting buffer (20mM-potassium phosphate, pH6.2). The bulk of enzyme could be eluted at a low concentration of KCl (20–100mM). Hydrogenase was strongly bound by Green A and Procion Red and released from both ligands with KCl over a concentration range of 0.2–1M. However, the elution profile with Green A was broader and the recovery of activity (60%) lower, so Procion Red (recovery: 75%) proved to be the most effective dye ligand for hydrogenase and was therefore selected for application in a larger-scale purification.

The purification procedure is described in detail in the Experimental section and summarized in Table 1. The most essential step was in fact the affinity chromatography on Procion Red–agarose. About 85% of total protein (2.5g) loaded on to a 500ml column did not bind to Procion Red and was recovered in the wash eluate (Fig. 1). Specific elution of hydrogenase with NAD was possible, but not satisfactory. Because the affinity of hydrogenase for NAD is relatively low (for K_m values, see under 'Inhibition of soluble hydrogenase by free Procion Red' below), a concentration of about 5mM-NAD was required to desorb hydrogenase quantitatively from the Procion Red column. As exposure to such a high NAD concentration causes destabilization of hydrogenase (K. Schneider, unpublished work), the enzyme became partially inactivated during elution. Therefore hydrogenase was preferably eluted by increasing ionic strength with a linear (0–1M) gradient of KCl. The fractions of highest hydrogenase activity were eluted at a concentration of 0.32M-KCl. This step gave about a 9-fold purification and an enzyme preparation of approx. 30% purity. Enzyme purity was, as in the following cases, estimated by polyacrylamide-gel electrophoresis in the presence and absence of SDS.

As a second column step, DEAE-Sephacel chromatography was employed under conditions essentially improved by comparison with previous procedures (Schneider & Schlegel, 1976; Schneider *et al.*, 1979). Instead of the use of a linear KCl gradient the salt concentration was increased stepwise. After extensive prewashings of the DEAE-Sephacel column with 50mM-potassium phosphate, pH7.0, and 70mM-KCl (in starting buffer), hydrogenase was selectively eluted as a homogeneous enzyme with 90mM-KCl. Elution with higher KCl concentrations resulted in higher recoveries, but with protein impurities. Therefore residual hydrogenase on the column was eluted with 150mM-KCl and pooled separately. This preparation was 85% pure.

The specific activity of the homogeneous 90mM-KCl eluate was 121 units (μmol/min per mg of protein (Table 1), more than twice the specific activity reported for homogeneous hydrogenase preparations in previous publications (Schlegel & Schneider, 1978; Schneider *et al.*, 1979). This high specific activity is suggested to be due to the ability of the Procion Red–agarose column to separate inactive from active enzyme. Inactive hydrogenase, which accumulates in the cell by inactivation *in vivo* (Schlesier & Friedrich, 1981) and probably during crude-extract preparation, binds to Procion Red–agarose with either diminished affinity or not at all. This was demonstrated by an independent experiment: a sample of hydrogenase purified by conventional methods (Schneider *et al.*, 1979) with a specific activity of only 30units/mg of protein was subjected to Procion Red–agarose chromatography. Although by the criterion of SDS/polyacrylamide-gel electrophoresis this hydrogenase was definitely homogeneous before Procion Red treat-

Table 1. *Purification of soluble hydrogenase from A. eutrophus H16*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Crude extract (soluble fraction)	6660	12614	1.89	1	100
Cetavlon treatment	5543	12604	2.27	1.2	100
$(NH_4)_2SO_4$ (40–60%-satd.)	2466	11291	4.58	2.4	90
Procion Red HE-3B–agarose	179	6825	38.02	20.1	54
DEAE-Sephacel					
90mM-KCl eluate	17.3	2095	121.00	64.0	17
150mM-KCl eluate	21.9	2545	116.34	61.5	20

ment, this step resulted in a 3-fold increase of specific activity.

A similar example for the resolving power of an affinity-chromatography technique was reported by Edmondson *et al.* (1972). The authors succeeded in the resolution of fully functional milk xanthine oxidase from non-functional enzyme by column chromatography on Sepharose-coupled pyrazolo-[3,4-*d*]pyrimidine. For this enzyme the inactive state was shown to be due to the lack of a persulphide group required for catalysis.

These and our own observations point to the general problem of mixtures of active and inactive enzyme in pure protein preparations and calls in question the reliability of physico-chemical analyses of proteins. Enzyme inactivation may arise already in whole cells during cultivation and harvesting, during cell breakage and also during each purification step. The consequence of such early inactivations often is that, up to the final preparation, an enzyme is accompanied by an inactive form of the same protein. This might be the reason for often-observed discrepancies in the content of constituents and cofactors of hydrogenases. For example, Erbes *et al.* (1975) determined for the 'ferredoxin-linked' hydrogenase of *Clostridium pasteurianum* 4–5 atoms each for iron and labile sulphur per enzyme molecule. In contrast, Chen *et al.* (1976) found, dependent on the specific activity of hydrogenase, 9–12 atoms for each element. For preparations of the soluble hydrogenase of *A. eutrophus* with lower specific activities (40–55 units/mg of protein) a content of 12 Fe atoms and 12 S^{2-} (Schneider *et al.*, 1979), and 1–2 FMN molecules (Schneider & Schlegel, 1978) per enzyme molecule has been calculated. It is our conclusion that, as there might be a correlation between the specific enzyme activity and the content of the enzyme's constituents, we have to re-examine the values for Fe, S and FMN with preparations of specific activities as high as that reported in this paper (121 units/mg of protein). The content of nickel, the newly discovered component of hydrogenase, has already been studied in preparations chromatographed on Procion Red–agarose (Friedrich *et al.*, 1982). Two atoms of nickel have been determined per molecule of pure hydrogenase.

Inhibition of soluble hydrogenase by free Procion Red

Kinetic studies with free Procion Red HE-3B confirmed that this dye inhibits hydrogenase by competing with NAD for the acceptor binding site. The inhibition effect of Procion Red was pH-dependent. At pH 8.0, 7.0 and 6.2, K_I values of 0.340, 0.103 and 0.025 mM respectively have been determined. These data fit well with the general observation that binding to immobilized triazine

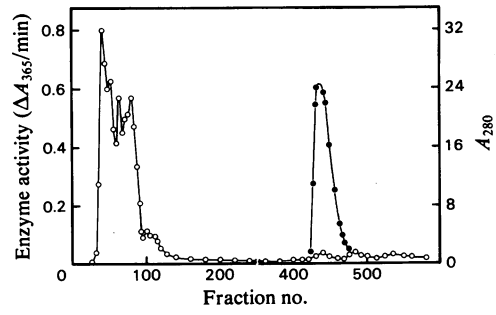

Fig. 1. *Elution profile of the soluble A. eutrophus hydrogenase from the Procion Red HE-3B–agarose column*

Protein (2.5 g) was applied to the column (5 cm × 60 cm) and eluted successively with starting buffer (50 mM-potassium phosphate, pH 6.2) and a linear KCl gradient (0–1 M). Fractions (6 ml) were collected. ○, Protein; ●, hydrogenase activity.

dyes increases on lowering the pH (Angal & Dean, 1978), presumably because of a contribution from ionic interactions favoured by increasing positive charges on interacting protein molecules (Dean & Watson, 1979). To be able to characterize the binding specificity of hydrogenase to Procion Red more conclusively, we also examined the K_m of hydrogenase for the substrate NAD at different pH values. The K_m values determined were 0.560 (pH 8.0), 0.213 (pH 7.0) and 0.141 (pH 6.2) respectively. These values perhaps suggest that the affinity of hydrogenase for NAD, in fact, changes in the same pH-dependent way as for Procion Red. This is remarkable and rather unexpected, as the pH optimum for NAD reduction of hydrogenase is at 8.0 and the activity is the more decreased the lower the pH value (Schneider & Schlegel, 1976). Even if the effect of pH on enzyme affinity is more pronounced with Procion Red than with NAD, the results suggest that non-specific interactions of hydrogenase with the dye only play a minor role.

Purification of membrane-bound hydrogenase from A. eutrophus

Surprisingly, the membrane-bound and electron-transport-chain-coupled hydrogenase of *A. eutrophus*, which does not react with nicotinamide nucleotides and which differs significantly from the cytoplasmic NAD-specific enzyme with respect to many properties (Schink & Schlegel, 1979), also exhibited a definite but somewhat weaker binding to some of the immobilized triazine dyes (Green A, Cibacron Blue, Procion Red). As for the cytoplasmic hydrogenase, the most favourable results were obtained with Procion Red HE-3B. Characteristic protein-elution and activity profiles of a preparative-scale chromatography on Procion Red–

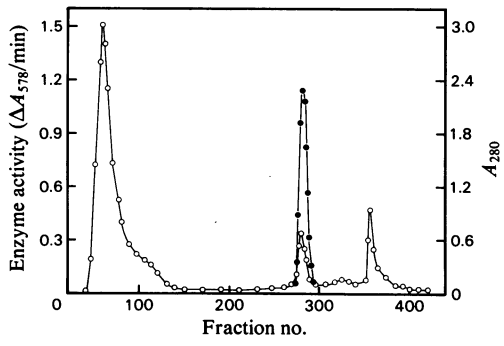

Fig. 2. *Elution profile of the membrane-bound A. eutrophus hydrogenase from the Procion Red HE-3B–agarose column*

The column (2.5 cm × 40 cm) was loaded with 0.7 g of protein, washed with 50 mM-potassium phosphate, pH 7.0, and then eluted with a linear KCl gradient (0–1 M). Fractions (4 ml) were collected. ○, Protein; ●, hydrogenase activity.

agarose are shown in Fig. 2. Although a relatively large proportion (~30%) of the total solubilized membrane proteins was bound to the dye, at a concentration of 0.23 M-KCl, hydrogenase was eluted with high selectivity as a sharp single peak. This step gave an approx. 6-fold purification. The resulting enzyme preparation was at least 95% pure and had a specific activity of 40.5 units/mg of protein (Table 2). The overall yield of enzyme activity amounted to 17%.

Inhibition of membrane-bound hydrogenase by free Procion Red

To find out whether Procion Red HE-3B binds specifically to the catalytic centre of the membrane-bound hydrogenase or whether binding is rather due to ionic and/or hydrophobic interactions, the effect of the free dye on enzyme activity was studied. It was found that hydrogenase is strongly inhibited by Procion Red, following a mechanism which appears to be largely competitive in character with respect to Methylene Blue (Fig. 3). With respect to H_2, inhibition was non-competitive. The K_I value determined for Procion Red in potassium phosphate buffer, pH 7.0, was 19 μM, which indicates, at least under reaction conditions, an even stronger affinity of the dye for this enzyme than for the NAD-reducing soluble hydrogenase of the same organism. In the case of the inosine 5′-monophosphate dehydrogenase of *Escherichia coli*, Procion Red HE-3B displayed, similarly to the soluble hydrogenase, a rather weak competitive inhibition versus NAD^+, with $K_I = 114.3\,\mu$M (Clonis & Lowe, 1981). For Cibacron Blue F3G-A, significantly lower K_I values, in the range of 0.1–1 μM, have often been reported for dehydrogenases (Wilson, 1976).

Binding of the hydrogenases from Alcaligenes latus and Clostridium pasteurianum on Procion Red–agarose

Because we considered it important to explore whether affinity chromatography on Procion Red is a method also applicable to hydrogenases of other organisms, we studied the binding behaviour on Procion Red–agarose of hydrogenases of two further micro-organisms, namely those of *Alcaligenes latus* and of *Clostridium pasteurianum*.

A. latus has been described as an aerobic N_2-fixing hydrogen bacterium (Malik *et al.*, 1981) and to belong to the family of Azotobacteriaceae rather than to the genus *Alcaligenes* (Malik & Claus, 1982). The hydrogenase isolated from this bacterium is an exclusively membrane-bound enzyme (Pinkwart *et al.*, 1983). The enzyme preparation resulting from solubilization and $(NH_4)_2SO_4$ treatment was found to bind to Procion Red–agarose even more tightly than the hydrogenases of *A. eutrophus*. However, the elution conditions used were less selective than for the latter enzymes. The main activity band was eluted from the column at a concentration of 0.48 M-KCl and a smaller enzyme fraction with lower binding specificity was eluted with only 0.34 M-KCl (result not shown). The 0.48 M-KCl eluate had a specific activity of 6.6 units/mg of protein and was about 25% pure. Details of the purification of the *A. latus* hydrogenase to homogeneity will be published elsewhere (Pinkwart *et al.*, 1983).

Clostridium pasteurianum has been described as containing two types of hydrogenases, the 'classical' reversibly functioning hydrogenase and a second so-called 'unidirectional' hydrogenase, which is unable to evolve H_2 from reduced electron carriers (Chen & Blanchard, 1978). Because of the oxygen-sensitivity of both enzymes, Procion Red–agar-

Table 2. *Purification of membrane-bound hydrogenase from A. eutrophus H16*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Crude membrane fraction	5562	5245	0.94	1	100
Solubilized membrane fraction	1872	4471	2.39	2.5	85
$(NH_4)_2SO_4$ (40–60%-satd.)	301	2104	7.00	7.4	40
Procion Red HE-3B–agarose	22.4	908	40.51	43.1	17

ose chromatography with the crude extract of *C. pasteurianum* was performed anaerobically under pure hydrogen. For protein elution a discontinuous KCl gradient was used (see the Experimental section). The results of this experiment (Table 3) undoubtedly demonstrated that both types of *Clostridium* hydrogenases bind to immobilized Procion Red. The two enzymes could clearly be differentiated by their different binding specificity and by their ability/inability to produce hydrogen. The bulk of the H_2-evolving hydrogenase was eluted from the column at a KCl concentration not lower than 1 M. In this fraction the specific activity (3.2 units/mg of protein) increased 4-fold compared with the activity in the crude extract (0.8 unit/mg of protein). A smaller proportion of this enzyme (~14%) was recovered in the unbound protein fraction (wash-buffer eluate). Non-binding of some enzyme molecules to Procion Red could be due to the masking of the catalytic centres by molecules of the physiological electron carrier ferredoxin still bound to the enzyme. The presence of ferredoxin in the wash-buffer eluate was demonstrated by the ability of this eluate to produce H_2 in the absence of Methyl Viologen. The reaction rate corresponded to about 20% of the rate obtained in the presence of Methyl Viologen.

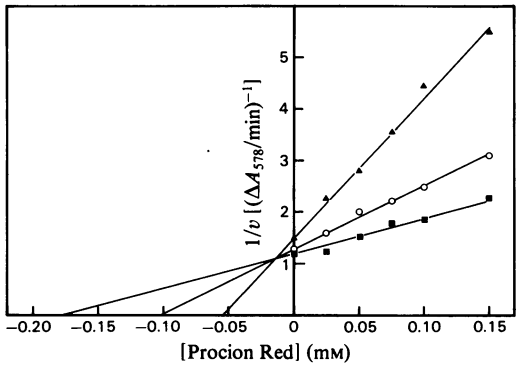

Fig. 3. *Dixon plot of inhibition of membrane-bound hydrogenase (A. eutrophus) by Procion Red HE-3B*
The reaction mixture (3 ml) contained 2 μg of protein, 50 mM-potassium phosphate, pH 7.0, 0.45 mM-H_2 and the following Methylene Blue concentrations: ▲, 0.1 mM; ○, 0.15 mM; ■, 0.2 mM. The concentration of Procion Red HE-3B was varied as indicated in the Figure.

The maximum H_2 uptake activity (Methylene Blue reduction) was detected in the 0.25 M-KCl eluate (Table 3). As this fraction did not show any H_2-evolving activity, it is concluded that the Methylene Blue reduction measured exclusively represented the unidirectional hydrogenase. Although this enzyme did bind to Procion Red more weakly than the 'reversible' hydrogenase, owing to a higher elution specificity and a 10-fold lower protein content, a comparatively high purification factor of 21 and a specific activity of 77.7 units/mg of protein was achieved.

Preliminary studies with the crude extract of *C. pasteurianum* on the interaction of the reversible hydrogenase with free Procion Red indicated a competitive inhibition of H_2 production with respect to reduced Methyl Viologen. In the presence of a constant concentration of Procion Red the percentage of inhibition did not change with varying H_2 concentrations, but it was higher the lower the Methyl Viologen concentration (Table 4). Evidence for competitive inhibition was also shown for the H_2-uptake activity, which, in the crude extract, comprised both hydrogenases. At 0.15 mM-Methylene Blue the Procion Red concentration for 50% inhibition was 40 μM.

Table 3. *Chromatography of the crude extract of Clostridium pasteurianum on Procion Red HE-3B–agarose*
Crude extract (37.4 mg) of *C. pasteurianum* was applied to a Procion Red HE-3B–agarose column (0.9 cm × 10 cm) and eluted with 50 mM-potassium phosphate, pH 6.2 (wash buffer) and then a discontinuous KCl gradient (concentrations indicated below). H_2 uptake activity was measured by monitoring the Methylene Blue reduction photometrically at 578 nm in 50 mM-Tris/HCl, pH 8.0; H_2 production activity was measured manometrically in 50 mM-potassium phosphate, pH 7.0. Further assay conditions were as given in the Experimental section.

		Total activity		Specific activity	
Protein fraction	Total protein (mg)	H_2 uptake (units)	H_2 production (units)	H_2 oxidation (units/mg of protein)	H_2 production (units/mg of protein)
Crude extract	37.4	137	29	3.67	0.78
Wash-buffer eluate	21.7	5.4	2.8	0.25	0.13
KCl eluate					
100 mM	1.0	2.0	0.6	2.00	0.60
250 mM	0.6	46.6	0	77.67	0
500 mM	0.8	12.5	0.6	15.63	0.75
1 M	6.2	36.8	19.7	5.94	3.18
2 M	5.1	0	0	0	0

Table 4. *Inhibition of the H_2 production activity by Procion Red*

H_2-production activity in the absence and presence (2.5 mM) of free Procion Red HE-3B was measured manometrically in 100 mM-potassium phosphate, pH 7.0. A 10 µl (~0.4 mg) portion of the crude extract of *Clostridium pasteurianum* was used for each assay. The concentration of Methyl Viologen was varied as indicated in the Table.

[Methyl Viologen] (mM)	Activity (µl of H_2 evolved/h) Without Procion Red	+ 2.5 mM-Procion Red	Inhibition (%)
20	795	435	45
10	690	270	61
5	480	30	94
2.5	312	0	100

Thompson *et al.* (1975) have claimed that Cibacron Blue F3G-A, the most established affinity dye ligand, functions as a nucleotide or coenzyme analogue and binds specifically to enzymes containing a dinucleotide fold. However, as the use of Cibacron Blue and other triazine dyes became widespread in recent years, it became obvious that these dyes are less specific than originally postulated (Dean & Watson, 1979; Lowe *et al.*, 1980). The list of proteins that may be purified by triazine-dye chromatography includes NAD^+- and $NADP^+$-dependent dehydrogenases, kinases, glycolytic enzymes, blood proteins and a number of seemingly unrelated proteins (Dean & Watson, 1979). More recent physico-chemical studies confirmed that the triazine dyes are not highly specific nucleotide analogues, since only part of the chromophore accurately mimics coenzyme binding to the complementary enzymes (Ashton & Polya, 1978; Biellmann *et al.*, 1979; Edwards & Woody, 1979).

Watson *et al.* (1978) described Procion Red HE-3B as a nucleotide-selective ligand with preferentially high affinity for NADP-requiring enzymes. However, at the present stage of information and knowledge about the limited specificity of triazine dyes and with respect to the finding that Procion Red also exhibits good affinity to NADP-independent proteins such as carboxypeptidase (Baird *et al.*, 1976) and inosine 5'-monophosphate dehydrogenase (Clonis & Lowe, 1981), the statement of Watson *et al.* (1978) rather appears to be an oversimplification. This is also confirmed by the results obtained in the present study. The NAD-specific cytoplasmic hydrogenase of *A. eutrophus* and indeed all other nicotinamide-nucleotide-independent hydrogenases examined, the two membrane-bound hydrogenases from *A. eutrophus* and *A. latus*, the reversible and the unidirectional type of hydrogenase from *C. pasteurianum* did bind to immobilized Procion Red with a relatively high level of binding specificity and were shown to be competitively inhibited by the free dye. The fact that the hydrogenases used comprised representatives of quite diverse types and the observation that Procion Red competed with Methylene Blue and Methyl Viologen, which are known as the most common electron carriers of hydrogenases (Adams *et al.*, 1981) suggest (i) that possibly all or most of the existing hydrogenases might contain a common electron-carrier binding site which bioselectively interacts with Procion Red, and (ii) that at least a certain part of the chromophore mimics the binding of the electron carrier to the enzyme. Although it is again clear from these statements that even a strong protein–triazine dye interaction does not necessarily imply the presence of a dinucleotide-fold, it cannot be ruled out that enzymes such as the clostridial hydrogenases and the membrane-bound hydrogenases of hydrogen bacteria, despite the fact that they are definitely not able to react with NAD or NADP, contain a nucleotide binding site. It has recently been demonstrated that the membrane-bound hydrogenase of *A. eutrophus* (H. G. Podzuweit, unpublished work) and *A. latus* (Pinkwart *et al.*, 1983) are able to reduce the flavin nucleotides FMN and FAD at acidic pH values. Khan *et al.* (1981) found that NAD and NADH have a stabilizing effect on the oxygen stability of the reversible ferredoxin-linked hydrogenase of *C. pasteurianum*. Those authors concluded that both NAD and NADH bind to the enzyme but fail to transfer electrons to/from the active centre.

From our findings and conclusions we expect that chromatography on Matrex-bound Procion Red or another even more suitable triazine dye is also an attractive method for the purification of other hydrogenases. As research on hydrogenase is also of practical significance (H_2 production by biophotolysis of water, regeneration of coenzymes) the improvement of purification procedures requiring only a few steps and yielding large amounts of enzyme is of particular interest.

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