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Isolation and immunological characterization of the four non-identical subunits of the soluble NAD-linked hydrogenase from *Alcaligenes eutrophus* H16

Klaus SCHNEIDER and Birgit PIECHULLA

Institut für Mikrobiologie, Universität Göttingen, Griesbachstrasse 8, 3400 Göttingen, and Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Strasse 3, 3400 Göttingen, FRG

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Summary — The soluble NAD-linked hydrogenase of *Alcaligenes eutrophus* H16 is a tetramer consisting of 4 non-identical subunits with molecular weights of 63 000, 56 000, 30 000 and 26 000. Conditions have been elaborated to separate and isolate each of these subunits as a single polypeptide by a preparative scale of polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS).

Against each of the 4 subunits, polyclonal antibodies were produced. From the crude sera isolated from rabbits, the antibodies (IgG fractions) were purified by Protein A-Sepharose chromatography.

By the double immunodiffusion method, comparison of the 4 types of subunits revealed that they are in fact different polypeptides. Subunit 1 ($M_r = 63\,000$) and subunit 2 ($M_r = 56\,000$) only reacted with their own specific antibodies and showed no cross-reaction whatsoever with the antibodies raised against the other subunits. The only immunological relationship among the different subunits was observed with subunit 3 ($M_r = 30\,000$) and subunit 4 ($M_r = 26\,000$); the type of cross-reaction indicated that they are partially identical.

A. eutrophus H16 contains, in addition to the soluble hydrogenase, a membrane-bound hydrogenase which is a dimer composed of 2 subunits with M_r of 61 000 and 30 000. Whereas the 2 native enzymes did not show any immunological cross-reaction with the respective antibodies, it was demonstrated by double immunofluorescence labeling on nitrocellulose filters that the larger subunit of the membrane-bound hydrogenase cross-reacted significantly with the antibodies raised against subunit 2 of the soluble hydrogenase. It is concluded that these two subunits are related to each other and differ only slightly with respect to structural properties.

The NAD-linked hydrogenase of *Nocardia opaca* 1b has a subunit structure identical to that of the soluble *Alcaligenes* hydrogenase. From the *Nocardia* enzyme, two dimers were isolated which have different reactivity, different subunit composition and no serological relationship. In accordance with their subunit composition, the larger, diaphorase-active dimer showed a distinct cross-reaction only with antibodies against subunits 1 and 3 of the soluble *Alcaligenes* hydrogenase, whereas the smaller, hydrogenase-active dimer cross-reacted significantly only with antibodies against subunits 2 and 4. All these reactions were of the partial identity type.

hydrogenase / subunits / antibodies / immunodiffusion / *Alcaligenes eutrophus*

Résumé — *L'hydrogénase soluble, liée au NAD, d'*Alcaligenes eutrophus *H16, est un tétramère constitué de quatre sous-unités non identiques dont les poids moléculaires sont : 63 000, 56 000, 30 000 et 26 000. Nous avons mis au point les conditions nécessaires pour séparer et isoler chacune de ces sous-unités sous forme*

d'un seul polypeptide pour chaque unité, par électrophorèse préparative sur gel de polyacrylamide en présence de dodécylsulfate de sodium (SDS).

Des anticorps polyclonaux ont été produits contre chacune des quatre sous-unités. Ces anticorps (fractions IgG), provenant de serums bruts isolés de lapins, ont été purifiés par chromatographie sur protéine-A-Sépharose.

La méthode de double immunodiffusion a permis de comparer les quatre types de sous-unités et a révélé qu'il s'agissait en fait de polypeptides différents. La sous-unité 1 (M_r = 63 000) et la sous-unité 2 (M_r = 56 000) ne réagissent qu'avec leurs propres anticorps spécifiques et ne montrent absolument aucune réaction croisée avec les anticorps dirigés contre les autres sous-unités. La seule relation immunologique observée entre les différentes sous-unités l'est entre la sous-unité 3 (M_r = 30 000) et la sous-unité 4 (M_r = 26 000); le type de réaction croisée indique que ces sous-unités sont partiellement identiques.

A. eutrophus *H16 contient, outre l'hydrogénase soluble, une hydrogénase membranaire qui est un dimère composé de deux sous-unités de M_r = 61 000 et 30 000. Alors que les deux enzymes natifs ne présentent aucune réaction immunologique croisée avec leurs anticorps respectifs, il a été démontré, par double marquage par immunofluorescence sur filtres de nitrocellulose, que la plus grande sous-unité de l'hydrogénase membranaire réagissait de façon significative avec les anticorps dirigés contre la sous-unité 2 de l'hydrogénase soluble. On en conclut que ces deux hydrogénases contiennent chacune une sous-unité étroitement apparentées et ne différant que légèrement quant à ses propriétés structurales.*

L'hydrogénase liée au NAD de Nocardia opaca *1b possède une structure en sous-unités identique à celle de l'hydrogénase soluble d'*Alcaligenes. *On a isolé de l'enzyme de* Nocardia *deux dimères qui ont une réactivité différente, une composition en sous-unités différente et n'ont pas de parenté sérologique. Conformément à leur composition en sous-unités, le plus grand dimère ayant une activité diaphorase ne montre une réaction croisée nette qu'avec les anticorps dirigés contre les sous-unités 1 et 3 de l'hydrogénase soluble d'*Alcaligenes, *alors que le plus petit dimère ayant une activité hydrogénase ne montre de réaction croisée significative qu'avec les anticorps dirigés contre les sous-unités 2 et 4. Toutes ces réactions croisées ne présentent qu'une identité partielle.*

***hydrogénase / sous-unités / anticorps / immunodiffusion* / Alcaligenes eutrophus**

Introduction

The type of NAD-linked hydrogenase which has been isolated and extensively studied from *Alcaligenes eutrophus* [1-6] and *Nocardia opaca* 1b [7, 8] was characterized as being a tetramer composed of four different types of subunits (M_r = 63 000, 56 000, 30 000, 26 000 [*A. eutrophus* hydrogenase]) [7]. Since this subunit structure is very unusual and since the formation of artificial fragments during long preparation procedures or during SDS electrophoresis cannot be completely ruled out, further evidence was required to confirm that the four described polypeptides were real subunits but of non-identical nature. One approach to elucidate the complex enzyme structure and to achieve a differentiation of subunits on aspects of function was the separation of the *Nocardia* hydrogenase into a diaphorase dimer with NADH-acceptor-oxidoreductase activity and a hydrogenase dimer with H_2 uptake and H_2 evolving activity in the presence of artificial electron carriers [8].

In this paper we describe the isolation of the four subunits of NAD-linked hydrogenase from *A. eutrophus* H16, the production of polyclonal antibodies against the denatured form of these subunits, and the immunological comparison of the isolated polypeptides. The subunit dimers of the *Nocardia* hydrogenase and the membrane-bound hydrogenase of *A. eutrophus* were also included in the comparative immunological studies.

Materials and methods

Chemicals

Acrylamide (4 × cryst.), sodium dodecylsulfate (2 × cryst.) and urea (analytical grade) were obtained from Serva, protein A-Sepharose from Pharmacia, the ion-retardation resin AG 11A8 from Bio-Rad, basic fuchsin, benzyl viologen and goat anti-rabbit IgG from Sigma. The sources of all other chemicals used were as listed in previous publications [1, 7-9].

Enzyme purification

Soluble and membrane-bound hydrogenase from *Alcaligenes eutrophus* H16 were isolated and purified as described by Schneider *et al.* [9]. Purification of the soluble hydrogenase of *Nocardia opaca* 1b and preparation of subunit dimers were performed as outlined in [7].

Isolation of subunits and electrophoresis procedures

The four subunits of the soluble, NAD-linked, hydrogenase from *A. eutrophus* H16 were isolated by a preparative scale of polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS), using a flat gel apparatus which has been constructed and described by Stegemann [10]. The gel slab had a thickness of 1 cm and contained 7.5 % acrylamide, 0.2 % SDS, and 8 M urea. The electrophoresis buffer used was 25 mM sodium phosphate, pH 7.0; 1 ml of the sample containing 15-20 mg purified hydrogenase and 1 % SDS was boiled for 3 min, mixed with sucrose (10 %) and then layered onto the gel surface. The electrophoresis was run at 80 mA and 90 V for 16 h. Then a thin slice of the gel was stained for protein in order to identify the location of the subunits. The regions which corresponded to the stained subunit bands were directly cut out of the gel and crushed by pressing through a syringe. The gel particles of each band were suspended in 20-30 ml of 100 mM sodium phosphate (pH 7.0) and the polypeptides eluted from the gel by stirring the suspension for 2 h. The four polypeptide solutions were separated from gel particles by filtration and then concentrated to about 1 ml each by ultrafiltration.

To examine the quality of separation, the preparations of the single subunits were analyzed by SDS electrophoresis of analytical scale. The conditions were the same as for preparative electrophoresis except that the run lasted only 2.5 h and was performed at a higher voltage (200 V). Protein was stained with Coomassie brilliant blue [11].

Removal of SDS from the subunit preparations

Residual amounts of SDS, which were present in subunit solutions and still bound to the polypeptides after their isolation, were removed by using the ion-retardation resin AG 11A8 [12]. The resin was washed with 5 vol of 1 M NH_4Cl, then with 20 vol of distilled water and was then equilibrated with 100 mM sodium phosphate buffer (pH 7.0). Small columns (0.5 × 3.5 cm) were prepared with 2 ml each of the pre-equilibrated resin; 0.5 ml each of the subunit solutions were then applied to the columns. Elution of the polypeptides was carried out with the equilibration buffer at a flow rate of 20-25 ml/h, and fractions of 0.5 ml were collected. The fractions containing protein were combined and concentrated to the original volume of 0.5 ml.

Determination of SDS content

The concentrations of SDS in subunit preparations were estimated by the procedure using basic fuchsin [13].

Protein determination

Proteins were determined by the method of Bradford [14].

Production and purification of antibodies

Antibodies against the native NAD-linked hydrogenase and against each of the four subunits of this enzyme were produced by immunization of rabbits, as described by Schink and Schlegel [15]. The rabbit IgG fractions were purified by protein A-Sepharose CL-4b chromatography [16].

Immunoassays

Immunodiffusion tests (Ouchterlony's procedure) were conducted in gels containing 1 % agarose in 50 mM diethylbarbiturate/acetate buffer, pH 8.2, on microscope slides [17].

The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose filters (western blotting) was carried out according to Towbin *et al.* [18]. The double immunofluorescence labeling of blotted proteins was done following the procedure of Kohring *et al.* [19].

Results and discussion

Isolation of the four subunits of NAD-linked hydrogenase

To obtain a preparation convenient for subunit isolation, the native NAD-linked hydrogenase from *Alcaligenes eutrophus* H16 was first purified to a homogeneous state [9]. Analytical polyacrylamide gel electrophoresis in the presence of SDS confirmed that this enzyme consists of four types of subunit forming two pairs of two closely neighbouring bands in the gel (Fig. 1, gel 1). The first pair of bands corresponds to subunits 1 and 2 and has molecular weights of 63 000 and 56 000, the second pair of bands corresponds to subunits 3 and 4 and has molecular weights of 30 000 and 26 000.

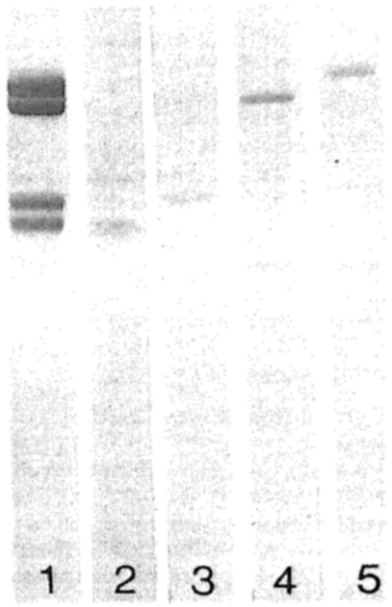

FIG. 1. — *SDS electrophoresis of the whole NAD-linked hydrogenase and of the isolated subunits.*

All gels contained 7.5 % acrylamide, 0.2 % SDS and 8 M urea. They were run for 2.5 h at 80 mA and 200 V. Gel 1, whole enzyme; gel 2, subunit 4; gel 3, subunit 3; gel 4, subunit 2; gel 5, subunit 1.

To find a method to split the enzyme into the four subunits under conditions as gentle as possible, several mild denaturing and chaotropic reagents (*e.g.*, urea, guanidine hydrochloride, trichloroacetate) and separation methods (HPLC gel filtration, sucrose density gradient centrifugation, chromatofocusing) were used; however, the only way to achieve full dissociation and clear separation of the subunits was the performance of a preparative SDS electrophoresis under conditions which have been optimized for analytical purposes [7] and turned out also to be most effective for preparative procedures. The gel (slab gel of 1 cm thickness) contained 7.5 % acrylamide, 0.2 % SDS and 8 M urea. It has to be emphasized that the acrylamide (4 × crystallized) and the SDS (2 × crystallized) used for electrophoresis were of special quality to avoid any negative and uncontrolled influence of reagent contaminants on the resolution power and also on the stability of the proteins. After the electrophoresis was run for 16 h at 80 mA and 100 V, the location of the subunits was identified by protein staining of a thin control slice of the gel. The migration distance of the subunits in the gel before isolation were : subunit 1 : 4.2 cm (R_f-value : 0.20); subunit 2 : 5.4 cm (R_f-value : 0.24); subunit 3 : 10.8 cm (R_f-value : 0.47); subunit 4 : 12.4 cm (R_f-value : 0.53). The regions corresponding to the subunit bands were directly cut out and eluted from the gel (see Materials and Methods). By subsequent analytical SDS electrophoresis the efficiency of the separation procedure was demonstrated. Each of the four subunits appeared to be homogeneous and migrated as a single band in the SDS gel (Fig. 1, gels 2-5). When about 20 mg protein were applied to preparative electrophoresis, the amount of protein isolated per run was 1.2-1.5 mg each for the two large subunits and 0.6-0.8 mg each for the two small subunits.

Immunological comparison of the four isolated subunits

The pure, denatured subunits obtained from preparative electrophoresis were used for the production of polyclonal, monospecific antibodies in rabbits. Before use in immunological studies, the antibodies (IgG fractions) were purified by chromatography on protein A-Sepharose.

Since it is known that the presence of SDS in protein preparations and in immunodiffusion gels strongly inhibits antibody-antigen reactions [20] and since we ourselves observed that in control experiments SDS (8 µl of 0.05-0.5 % solutions/well) reacted with antibodies to give weak and diffuse but disturbing stainable bands (not shown), we removed the SDS from protein and protein solutions by special treatment with the ion-retardation resin AG 11A8 (see Materials and Methods). Determination of the SDS content (assay with basic fuchsin [13]) in the subunit preparations before and after treatment with the resin revealed that we succeeded in completely removing SDS. The original subunit samples contained SDS concentrations varying from 0.06 to 0.11 %, whereas after resin treatment the SDS concentration in each sample was zero. For immunological studies only the SDS-free subunit preparations were used.

Our main method to analyze the serological relationship between proteins was the comparative double diffusion technique. To be able to directly compare all the four isolated subunits, the gel wells of an agarose plate were filled according to the following system : The centre well contained the antibodies directed against one subunit (anti-subunit immunoglobulins) and two

neighbouring wells each contained the subunits to be compared.

Subunit 1 only reacted with its own specific antibodies. If two samples of subunit 1 were placed in two neighbouring wells (Fig. 2, gel A, wells 1, 2) the resulting precipitation lines completely fused documenting the identity of the protein samples. When samples of subunit 1 were placed in wells distant from each other (5, 7, 11 in gel A of Fig. 2) and one each of the subunits 2, 3 and 4 were placed in the respective neighbouring wells (4, 6, 10), only straight, single precipitation bands of subunit 1—anti-subunit 1 immunoglobulin (IgG) reactions occurred, whereas the other subunits did not show any cross-reaction.

Exactly the same precipitation pattern was obtained with subunit 2, when the centre well contained the anti-subunit 2 IgG (Fig. 2, gel B). These antibodies only reacted with subunit 2 itself, but showed no cross-reaction at all with subunits 1, 3 and 4.

A different behaviour was demonstrated with subunits 3 and 4 and their antibodies. The anti-subunit 3 IgG did not cross-react with subunits 1 and 2 but showed a distinct cross-reaction with subunit 4. The reaction type was that of partial fusion of the precipitation lines with the formation of a single spur (Fig. 2, gel C, wells 10, 11) indicating that subunits 3 and 4 are partially identical proteins. This was confirmed by the fact that, *vice versa*, the anti-subunit 4 IgG cross-reacted with subunit 3, also exhibiting, although the reaction type appeared to be less pronounced, partial identity (Fig. 2, gel D, wells 10, 11). Subunits 1 and 2 did not cross-react with anti-subunit 4 IgG.

The conclusion from these results is that the four isolated polypeptides from the NAD-linked hydrogenase are in fact different types of subunits. Subunits 1 and 2 do not have any serological relationship, neither with each other, nor with subunits 3 and 4. Only the smaller subunits 3 and 4 are related to each other. They are not identical but contain a certain number of common antigenic determinants.

Immunological characterization of the subunit dimers of the hydrogenase from Nocardia opaca *1b*

The NAD-linked hydrogenase of *N. opaca* 1b has been described as being identical to the

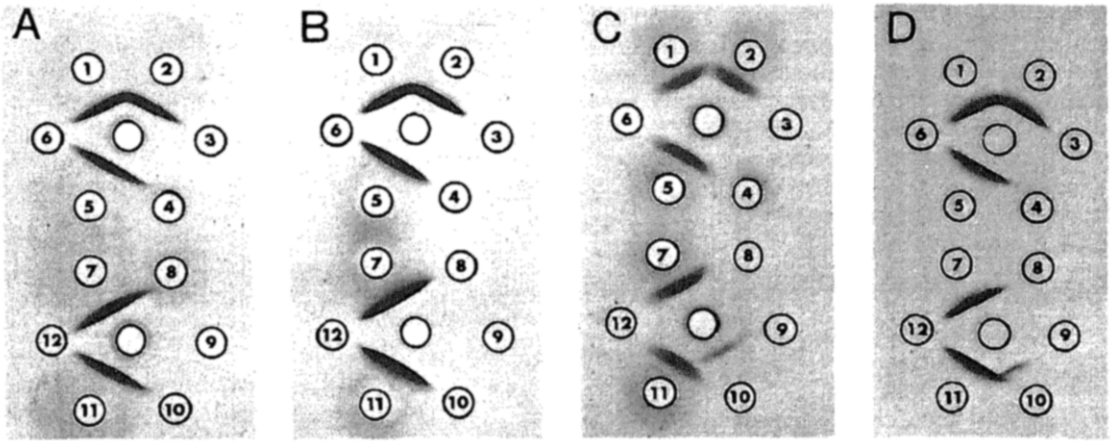

FIG. 2. — *Immunological comparison of the subunits of the NAD-linked hydrogenase by double diffusion.*

The agarose gels were prepared as described in Materials and Methods. Gel A : both centre wells contained 0.95 mg anti-subunit 1 IgG, wells 1, 2, 5, 7, 11 each contained 3.1 µg of subunit 1, well 4 3.8 µg of subunit 2, well 8 6.8 µg of subunit 3 and well 10 4.5 µg of subunit 4; wells 3, 6, 9, 12 remained empty. Gel B : both centre wells contained 0.31 mg anti-subunit 2 IgG, wells 1, 2, 5, 7, 11 each contained 3.8 µg of subunit 2, well 4 3.1 µg of subunit 1, well 8 6.8 µg of subunit 3 and well 10 4.5 µg of subunit 4; wells 3, 6, 9, 12 remained empty. Gel C : both centre wells contained 0.77 mg anti-subunit 3 IgG, wells 1, 2, 5, 7, 11 each contained 9 µg of subunit 3, well 4 4.2 µg of subunit 1, well 8 5 µg of subunit 2 and well 10 6 µg of subunit 4; wells 3, 6, 9, 12 remained empty. Gel D : both centre wells contained 0.63 µg anti-subunit 4 IgG, wells 1, 2, 5, 7, 11 each contained 45 µg of subunit 4, well 4 3.1 µg of subunit 1, well 8 3.8 µg of subunit 2 and well 10 6.8 µg of subunit 3; wells 3, 6, 9, 12 remained empty. Immunodiffusion was performed in a humid atmosphere at room temperature for 24 h. The gel slabs were washed for 24 h in physiological saline and subsequently stained with Coomassie brilliant blue.

NAD-linked hydrogenase of *A. eutrophus* with respect to subunit structure and cofactor composition, and partially identical with respect to immunological properties [7, 8]. The *N. opaca* hydrogenase is unique insofar as, at low ionic strength, alkaline pH values, and in the absence of $NiCl_2$, it dissociates into two subunit dimers : one larger dimer which consists of subunits 1 and 3 and has diaphorase (NADH-acceptor-oxidoreductase) activity but is completely hydrogenase inactive, and one smaller subunit dimer which consists of subunits 2 and 4 and has high hydrogenase activity. It does not react with NAD but catalyzes H_2 uptake and H_2 evolution in the presence of artificial electron carriers [8].

Antibodies against the two dimers are not yet available; however, for a preliminary immunological characterization and differentiation of the dimers, the present antibodies against whole NAD-linked hydrogenase of *A. eutrophus* and against the single subunits of this enzyme appeared to be quite suitable.

Using the antibodies against the whole enzyme, depending on the combination of proteins, all three basic reaction types could be demonstrated (Fig. 3) : As expected, the undissociated tetramer was only partially identical with each of the dimers (Fig. 3, gel B). When the diaphorase and the hydrogenase dimer were compared directly, the precipitation pattern proved that they are non-identical serologically unrelated proteins : both dimers are part of the whole enzyme and therefore both contain, of course, antibody binding sites; however, these binding sites are of different specificity, resulting in two precipitation lines which did not fuse at all, but crossed each other forming two spurs (Fig. 3, gel C).

As controls, two samples of each of the same dimer were also compared : they gave the characteristic patterns of fully identical proteins (Fig. 3, gel A). Generally, the reactions of the hydrogenase dimer resulted in sharper and more intense bands.

Comparison of the two dimers with the single subunits separated from the *A. eutrophus* hydrogenase showed the following results : The diaphorase dimer (subunits 1+3) reacted partially identically with subunits 1 and 3 (Fig. 4, gel A), very weakly with the antibodies against subunit 4 (Fig. 4, gel B, wells 1-6) and not at all with the antibodies against subunit 2. *Vice versa,* the hydrogenase dimer (subunits 2 and 4) reacted partially identically with the isolated subunits 2 and 4 (Fig. 4, gel C), whereby the reaction with subunit 4 indicated near identity, forming only a very weak spur (Fig. 4, gel C, wells 7-12). With the antibodies raised against subunits 1 and 3, the hydrogenase dimer did not react or, in the case of anti-subunit 3 IgG, reacted extremely weakly, but only when several-fold amounts of antibodies were applied (Fig. 4, gel B, wells 7-12).

The results are in very good accordance with (i) the postulated subunit composition of the dimers [7] and (ii) the preceding immunological analyses of this work which state the great diversity of the subunits. The described weak reactions (Fig. 4, gels B, C) are obviously due to the partial identity of subunits 3 and 4. The reason(s) for the extreme weakness of the reactions might be that the immunological relation-

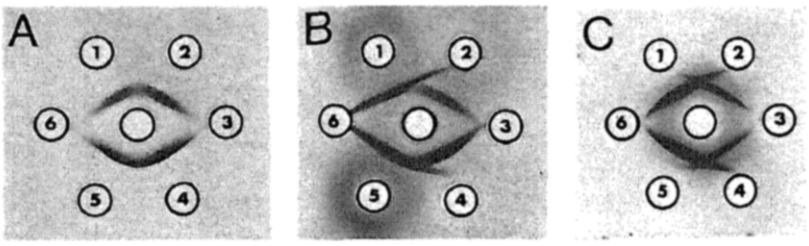

FIG. 3. — *The subunit dimers of hydrogenase from* N. opaca : *Immunological comparison with each other and with the whole enzyme.*

Experimental conditions were as described for Figure 3. Gel A : the centre well contained 0.54 mg anti-hydrogenase (soluble enzyme, *A. eutrophus*) IgG, wells 1 and 2 each contained 18 µg of the diaphorase dimer, wells 4 and 5 each contained 10 µg of the hydrogenase dimer, wells 3 and 6 remained empty. Gel B : the centre well contained 0.54 mg anti-hydrogenase (soluble enzyme, *A. eutrophus*) IgG, wells 1 and 5 each contained 30 µg of the whole hydrogenase *(N. opaca),* well 2 18 µg of the diaphorase dimer and well 4 10 µg of the hydrogenase dimer; wells 3 and 6 remained empty. Gel C : the centre well contained 0.77 mg anti-hydrogenase (soluble enzyme, *A. eutrophus*), wells 1 and 5 each contained 10 µg of the hydrogenase dimer and wells 2 and 4 18 µg each of the diaphorase dimer; wells 3 and 6 remained empty.

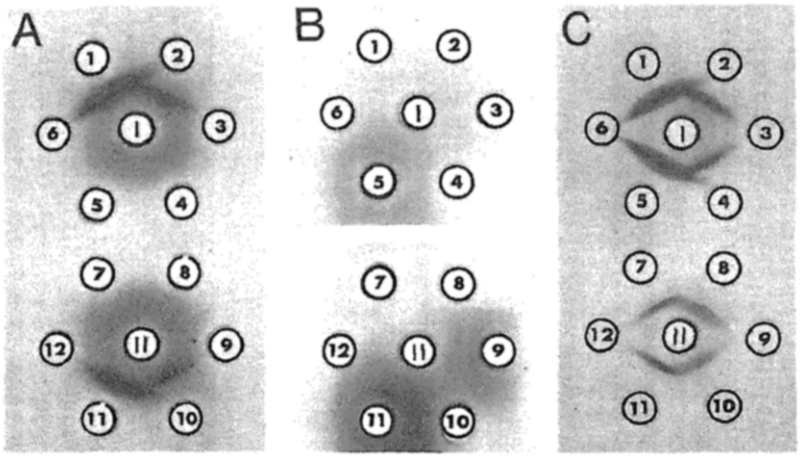

FIG. 4. — *Immunological comparison of the subunit dimers of* Nocardia *hydrogenase with the single subunits of the* Alcaligenes *hydrogenase.*

Gel A : centre well I contained 1.9 mg anti-subunit 1 IgG, well 1 contained 6.2 µg of subunit 1 and well 2 12 µg of the diaphorase dimer; centre well II contained 2.15 mg anti-subunit 3 IgG, well 10 contained 12 µg of the diaphorase dimer and well 11 9 µg of subunit 3; wells 3-6, 7-9 and 12 remained empty. Gel B : centre well I contained 12 µg of the diaphorase dimer and wells 1, 3, 5 contained 0.28, 0.56 and 1.12 mg of anti-subunit 4 IgG, respectively; centre well II contained 20 µg of the hydrogenase dimer and wells 1, 3, 5 contained 0.51, 1.02 and 2.04 mg of anti-subunit 3 IgG, respectively; wells 2, 4, 6, 8, 10, 12 remained empty. Gel C : centre well I contained 0.82 mg of anti-subunit 2 IgG, wells 1 and 5 each contained 10.4 µg of subunit 2 and wells 2 and 4 10 µg each of the hydrogenase dimer; centre well II contained 0.41 mg anti-subunit 4 IgG, wells 7 and 11 contained 3 µg each of subunit 4 and wells 8 and 10 24 µg each of the hydrogenase dimer; wells 3, 6, 9, 12 remained empty.

ship between subunits 3 and 4 is : (i) less pronounced in the native than in the denatured proteins, and/or (ii) is less pronounced in the *Nocardia* enzyme than in the *Alcaligenes* enzyme.

Immunological comparison of soluble and membrane-bound hydrogenase of A. eutrophus *H16*

A. eutrophus H16 contains, in addition to the soluble NAD-linked hydrogenase (SH), a membrane-bound hydrogenase (MBH) coupled to the aerobic respiratory chain [21]. These two enzymes have been described as being significantly different with respect to most of the catalytic, molecular and immunological properties [15, 22]. The MBH is a dimer composed of two subunits with molecular weights of 61 000 and 30 000. In our work we confirmed the earlier finding [15] that the two hydrogenases in their native form did not show any immunological cross-reaction with the respective antibodies. It was, however, still an open question whether both enzymes, in spite of demonstrated structural and immunological diversity, contain a common or closely related subunit which exhibits immunological relationship only in the isolated and/or denatured state. We therefore compared, in a further immunological study, the single subunits of the SH with the single subunits of the MBH, using the antibodies raised against the four subunits of the SH. Since we did not have preparations of isolated MBH subunits, we applied the method of double immunofluorescence labeling of blotted proteins [18]. This method enable direct use of the purified, native MBH which in the first step of the experiment was separated into subunits by SDS electrophoresis. The subunit proteins were then transferred from SDS gels onto nitrocellulose filters by western blotting, and immunologically detected with fluorescein isothiocyanate-labeled antibodies.

In fact, we were able to provide evidence that two subunits, one of either enzyme, are related to each other. These are subunit 2 ($M_r = 56\,000$) of the NAD-linked hydrogenase and the larger subunit ($M_r = 61\,000$) of the MBH. This latter subunit showed a distinct cross-reaction with the anti-subunit 2 IgG. The cross-reaction was visualized by fluorescence under ultraviolet light (254 nm) yielding a fluorescence band at the site where the large MBH subunit was located (Fig. 5, nitrocellulose filter 4). The fluorescence band of subunit 2 (SU2) is also presented as reference (Fig. 5, filter 3). It has, corresponding to a slightly lower molecular weight, migrated a bit further

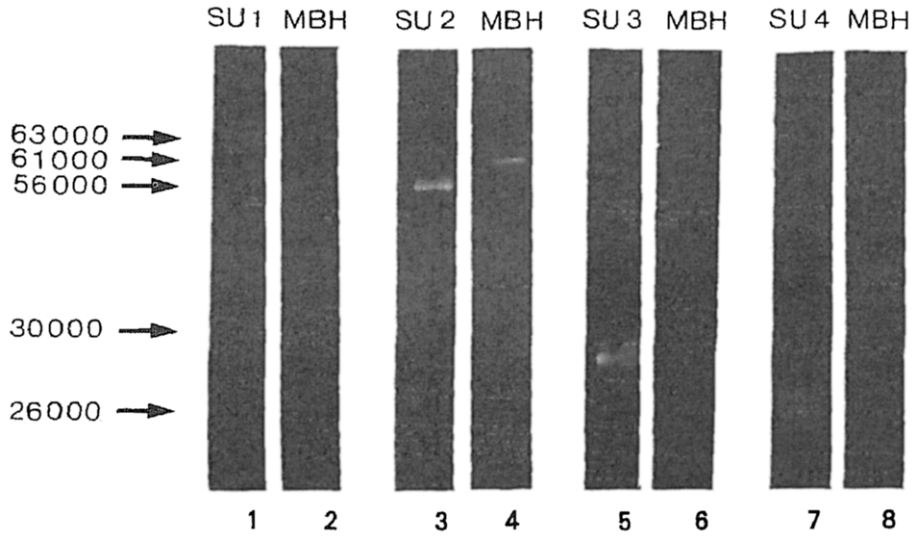

FIG. 5. — *Double immunofluorescence of blotted subunits of soluble and membrane-bound hydrogenase.*

The isolated subunits (10-15 µg each) from the soluble hydrogenase and the whole enzyme (30 µg) from the MBH were applied to SDS electrophoresis. The electrophoretically separated subunits were transferred onto nitrocellulose filters by western blotting [18]. The nitrocellulose filters were incubated (room temperature) 12 h each with solutions containing the specific antibodies against the subunits (1 µg IgG each/ml buffer) and subsequently with a solution containing goat-anti-rabbit IgG (3 µg/ml buffer) labeled with fluorescein isothiocyanate (FITC). The immunologically reactive subunits were detected by fluorescence under UV light (254 *nm*). Nitrocellulose filters 1, 3, 5, 7 contained the single subunits (SU) of the soluble enzyme, as indicated in the figure and the filters 2, 4, 6, 8 each contained the subunits of the MBH. Filters 1 and 2 were incubated in the presence of anti-SU 1 IgG, filters 3 and 4 in the presence of anti-SU 2 IgG, filters 5 and 6 in the presence of anti-SU 3 IgG and filters 7 and 8 in the presence of anti-SU 5 IgG.

than the MBH subunit during electrophoresis. No fluorescence was detectable when the nitrocellulose filters which contained the MBH subunits were treated with antibodies directed against subunits 1, 3 and 4 (see Fig. 5, filters 2, 6, 8 and references **1, 5, 7**). From these results we conclude that the larger MBH subunit is only related to subunit 2 of the soluble enzyme, but not to subunits 1, 3 and 4, and that the smaller MBH subunit has no relation to any of these subunits. Estimation of the degree of relationship between subunit 2 (SH) and the large subunit (MBH) must await the availability of the MBH subunit in an isolated state and antibodies against this subunit. The SH subunit and the MBH subunit might be closely related to each other; however, complete identity seems rather improbable, since the two subunits differ at least with respect to their molecular size.

Nickel and one Fe-S cluster (probably a [4Fe-4S] cluster) have been demonstrated to be present in the hydrogenase-active dimer of the *Nocardia* hydrogenase and have been postulated to be essential components for the hydrogenase catalytic function [8]. Recently, a protein of a hydrogenase-defective mutant of *A. eutrophus* was isolated and immunologically analyzed as being identical with subunit 2 of the NAD-linked hydrogenase (Hornhardt, Schneider and Schlegel, this volume). This mutant protein contained both nickel and one Fe-S cluster and showed hydrogenase activity, even though the activity was very low because of the lability of this protein in the isolated state. It may therefore be concluded that subunit 2 of the NAD-linked hydrogenase and the large subunit of the membrane-bound hydrogenase represent the real basic hydrogenase in which the H_2 activation centre and the hydrogenase-specific cofactors are located. The function of the small subunit (subunit 4) in the hydrogenase-active dimer of the soluble enzyme apparently is not to participate in the reaction but to stabilize the structure of the active subunit 2. In the MBH dimer the smaller subunit may play another or an additional role. The MBH contains in addition to nickel not only one Fe-S cluster but at least two Fe-S clusters [23]. An Fe-S cluster might, with respect to this enzyme, also be located in the small subunit, and possibly has a specific function in transferring electrons to the physiolo-

gical electron acceptor. To prove this, future studies will have to deal with the isolation and characterization of intact subunits.

Acknowledgments

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