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Crosslinking and radiation inactivation analysis of the subunit structure of the pyridine nucleotide transhydrogenase of *Escherichia coli*

Cynthia Hou [1], Michel Potier [2] and Philip D. Bragg [1]

[1] *Department of Biochemistry, University of British Columbia, Vancouver and* [2] *Section de Génétique Médicale, Hôpital Sainte-Justine, Université de Montréal, Montréal (Canada)*

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The pyridine nucleotide transhydrogenase of *Escherichia coli* consists of two types of subunit (α: M_r 53906; β: M_r 48667). The purified and membrane-bound enzymes were crosslinked with a series of bifunctional crosslinking agents and by catalyzing the formation of inter-chain disulfides in the presence of cupric 1,10-phenanthrolinate. Crosslinked dimers α_2, $\alpha\beta$ and β_2, and the trimer $\alpha_2\beta$ were obtained. A small amount of tetramer, probably $\alpha_2\beta_2$, was also formed. Radiation inactivation was used to determine the molecular size of the transhydrogenase. The radiation inactivation size (217000) and chemical crosslinking are consistent with the structure (M_r 205146) being the oligomer that is responsible for biological activity.

Introduction

Pyridine nucleotide transhydrogenase, found in the cytoplasmic membrane of *Escherichia coli* and in the inner membrane of mitochondria, catalyzes the reversible transfer of a hydride ion equivalent between NAD and NADP [1]. Evidence has been accumulated from studies of the mitochondrial and *E. coli* transhydrogenase that this enzyme functions as a proton pump and translocates protons across the membrane according to the equation [1,2]:

$$n\mathrm{H}^+_{\mathrm{in}} + \mathrm{NADPH} + \mathrm{NAD} \leftrightarrow n\mathrm{H}^+_{\mathrm{out}} + \mathrm{NADP} + \mathrm{NADH}$$

Recently we have substantially increased our knowledge of the *E. coli* transhydrogenase by cloning and sequencing the *pnt* (pyridine nucleotide transhydrogenase) gene [2–4], and by purifying the enzyme and reconstituting it as a proton pump in phospholipid vesicles [5]. The enzyme consists of two types of subunits, α(M_r 53906) and β(M_r 48667). At present there is no information concerning the subunit structure of the enzyme or of its functional size.

In the present paper, we have investigated the molecular size of the transhydrogenase in situ in the membrane taking advantage of a strain of *E. coli* containing a multicopy plasmid which overproduces the enzyme to become the major protein of the cell membrane. Chemical crosslinking and radiation inactivation analysis indicated that the functional unit of the transhydrogenase has an $\alpha_2\beta_2$ subunit structure.

Materials and Methods

Preparation of transhydrogenase

Membranes (everted inner membrane vesicles) were prepared from the *E. coli* strain JM83 carrying the *pnt* gene on the multicopy plasmid pDC21 as described by Clarke and Bragg [5]. The membranes were suspended at a protein concentration of 3–4 mg/ml in 50 mM Tris/5 mM $MgSO_4$/1 mM dithiothreitol/1 mM EDTA and adjusted to pH 7.8 with H_2SO_4. The samples for radiation inactivation were lyophilized and reconstituted after irradiation by addition of water to obtain

Abbreviations: SDS, sodium dodecyl sulfate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DEB, 1,2 : 3,4-diepoxybutane; DMA, dimethyl adipimidate; DMS, dimethyl suberimidate; DSP, 3,3′-dithiobis(succinimidyl propionate); DST, disuccinimidyl tartarate; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); PAGE, polyacrylamide gel electrophoresis.

Correspondence: P.D. Bragg, Department of Biochemistry, University of British Columbia, 2146 Health Sciences Mall, Vancouver, B.C., Canada V6T 1W5

the original concentrations of buffer components. The transhydrogenase was solubilized and purified from membranes as described previously [5]. The assay of energy-independent transhydrogenase activity is described in Ref. 5.

Crosslinking

Membranes for crosslinking were stripped of extrinsic proteins with 1% (v/v) Triton X-100 and 2% sodium cholate as described by Clarke and Bragg [5]. The stripped membranes were freed of detergent by sedimentation from 50 mM Tris/1 mM EDTA/1 mM dithiothreitol and adjusted to pH 7.8 with HCl. They were suspended for crosslinking at a protein concentration of 5 mg per ml in 50 mM triethanolamine/20% (v/v) glycerol and adjusted to pH 7.5 with HCl. Crosslinking by cupric 1,10-phenanthrolinate was carried out as described previously [6]. Crosslinking by the other agents, and one- and two-dimensional SDS-PAGE, followed the procedures described in Ref. 7.

Radiation inactivation

Membrane samples were irradiated in triplicate at 38°C from a ^{60}Co source in a Gammacell model 220 instrument (Atomic Energy of Canada, Ottawa) at a dose rate of about 1 Mrad/h. Appropriate controls for non-irradiated preparations were run concurrently under the same conditions, but without irradiation. The following empirical equation [8] was used to relate molecular size to $D_{37,t}$, the radiation dose (in Mrad) necessary to inactivate the enzyme to 37% of its initial value, and to t, the temperature (in °C):

$$\log M_r = 5.89 - \log D_{37,t} - 0.0028\, t$$

$D_{37,t}$ values were obtained from a semi-logarithmic plot of specific activity of transhydrogenase versus radiation dose by using a least-squares fit. A similar equation was used to estimate target size.

Results

E. coli JM83 containing the plasmid pDC21 overproduces the transhydrogenase, such that the enzyme is the major protein of the cell membrane. This is clearly seen in Fig. 1, lane 8, where membranes have been submitted to SDS-gel electrophoresis and stained for protein with Coomassie blue. The α and β subunits of the enzyme are very evident. The high concentration of enzyme protein permitted crosslinking and radiation inactivation analyses to be conducted directly with membrane preparations.

Chemical crosslinking of transhydrogenase

Membranes for crosslinking were stripped of extrinsic proteins with detergent (see Materials and Methods). In the stripped membranes the transhydrogenase sub-

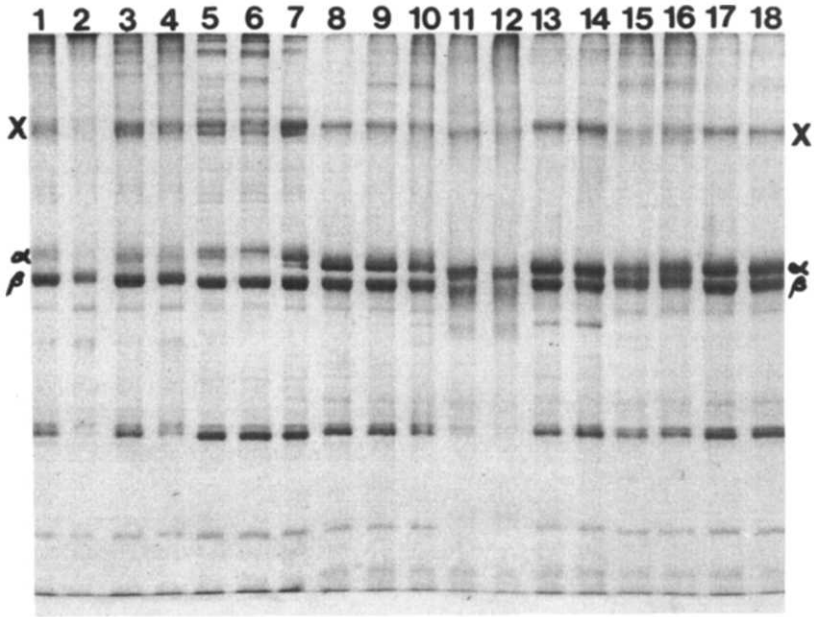

Fig. 1. One-dimensional SDS-PAGE of crosslinked membrane-bound transhydrogenase. The positions of migration of subunits and of crosslinked dimers (X) are shown at the side of the gel. The samples in lanes 1–7 were run in the absence of 2-mercaptoethanol. Lanes 8–18 were run in the presence of the thiol. Lanes 7, 8 contain noncrosslinked membranes. The remaining lanes contain enzyme crosslinked as follows: 1, 2: 0.25 mM, 0.75 mM DSP; 3, 4: 0.25 mM, 0.75 mM DTSSP; 5, 6: 0.063 mM, 0.19 mM cupric 1,10-phenanthrolinate; 9, 10: 2.9 mM, 8.7 mM DST; 11, 12: 5 mM, 15 mM EDC; 13, 14: 0.1 M (1.5 h), 0.1 M (3 h) DEB; 15, 16: 3.7 mM, 11 mM DMS; 17, 18: 4.1 mM, 12.2 mM DMA. Crosslinking by DSP and DTSSP was carried out for 15 min. The other agents (except as noted) were allowed to react for 3 h. The reaction with DEB was carried out at 37°C. The other reactions were at 22°C. The concentration of polyacrylamide in the gel was 9% (w/v). The gel was stained with Coomassie blue.

units composed about 70% of the membrane protein, as determined by scanning of stained gels. As shown in Fig. 1, lane 8, SDS-PAGE of membranes revealed a small amount of a material migrating in the position marked X on the gel, besides the α and β subunits of the transhydrogenase, and small amounts of other membrane proteins. The rate of migration is characteristic of a species of molecular weight about 100 000. It has been shown previously to arise by nonspecific and irreversible aggregation of subunits when the transhydrogenase is dissolved in electrophoresis sample buffer [3]. The sample in lane 8 was run in the presence of 2-mercaptoethanol. If this compound was omitted, then a larger amount of material was found as a doublet in the band X region (Fig. 1, lane 7). Furthermore, the α-subunit migrated as a doublet and was in lower amounts compared with the sample run with 2-mercaptoethanol. The doublet is due to the formation of internal disulfide bonds between cysteine residues in the α subunit. A somewhat similar pattern was observed in samples treated with cupric 1,10-phenanthrolinate. New bands migrating with molecular weights greater than 100 000 were evident also. Most of the crosslinking agents examined gave species which migrated in the region of band X (Fig. 1) as well as higher molecular weight species. In a number of instances, particularly with DSP and DTSSP (Fig. 1, lanes 1–4), this was accompanied by a reduction in the amount of the α subunit. However, EDC showed preferential modification of the β subunit.

The products formed by crosslinking with cupric 1,10-phenanthrolinate were more clearly resolvable on SDS polyacrylamide gels than those given by the other crosslinking agents. Thus, it was decided to study crosslinking with the agent in more detail. Moreover, this agent results in 'zero-length' crosslinking [9] between two sulfhydryl groups in proximity in the enzyme molecule, and the crosslinking can be readily reversed by reduction with thiols such as dithiothreitol and 2-mercaptoethanol. The difficulty of reversal of crosslinking by some of the other agents prevented easy analysis of the products of crosslinking. Our efforts to reverse crosslinking by EDC, using a previously successful method [7], were ineffective. This was regrettable since the pattern of crosslinking by EDC differed from that of the other agents (Fig. 1).

Analysis of the subunits crosslinked by cupric 1,10-phenanthrolinate was carried out as follows (Fig. 2B). The products of crosslinking were resolved by SDS-PAGE in one dimension (as in the stained gel strips mounted above the larger gels in Fig. 2). An unstained strip from the first-dimension gel was mounted horizontally in agarose containing 2-mercaptoethanol above a polyacrylamide gel slab. Electrophoresis into the second dimension of the subunits released by reduction showed previously crosslinked subunits migrating off the diagonal path travelled by noncrosslinked subunits. The components which were previously crosslinked together now migrated vertically above one another in the second dimension.

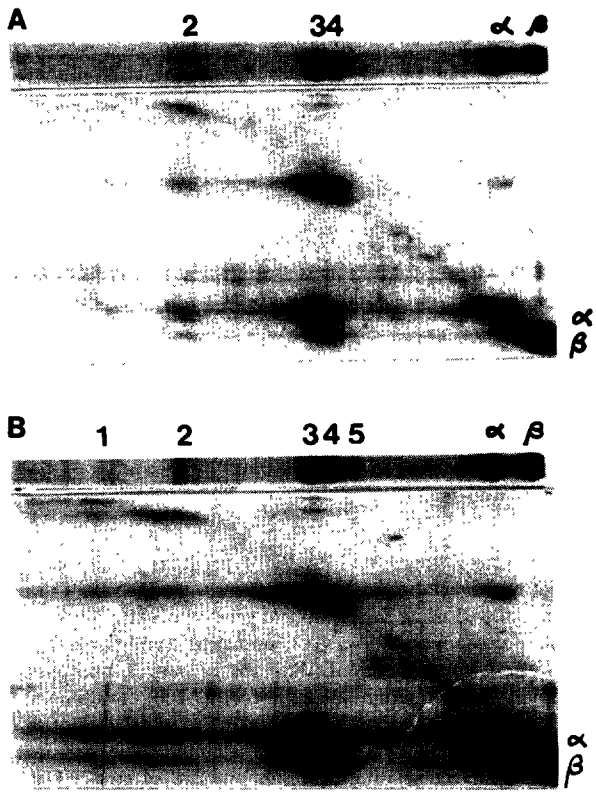

Fig. 2. Two-dimensional SDS-PAGE of membrane-bound transhydrogenase crosslinked with cupric 1,10-phenanthrolinate (B). (A) Untreated enzyme. The direction of migration is from left to right in the first dimension and from top to bottom in the second dimension. A stained duplicate strip of the first-dimension gel (5% polyacrylamide) is placed above the two-dimensional gel (9% polyacrylamide) for identification of crosslinked products (1–5). The subunits arising from cleavage of these products by 2-mercaptoethanol are seen in the two-dimensional gel vertically below the labeled bands. The positions of migration of the subunits of the transhydrogenase are indicated.

The stained first-dimension gel strip in Fig. 2B shows several groups of crosslinked subunits (1–5) as well as uncrosslinked α and β subunits. Species 2, 3 and 4 are clearly evident in the first-dimension gel of the untreated membranes (Fig. 2A) and undoubtedly arise from spontaneously-induced crosslinking of suitably positioned cysteine residues. (Note that the first-dimension gel was run without 2-mercaptoethanol.) Examination of the second-dimension gels shows that bands 3 and 4 are α_2 and $\alpha\beta$ crosslinked species. Band 5 is due to β_2. Both species would be expected to run with an apparent molecular weight of about 100 000. The second-dimension gel of Fig. 2A shows evidence (double spots) of some crosslinking between cysteine residues in the same α subunit. Note the relatively large amount of irreversibly aggregated subunits of M_r 100 000 migrating on the diagonal.

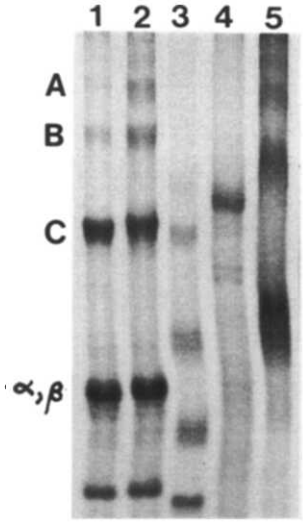

Fig. 3. One-dimensional SDS-PAGE of products arising by crosslinking of membrane-bound transhydrogenase by DTSSP. The products were resolved using the phosphate gel system. The concentration of polyacrylamide was 5% (w/v). 1, untreated membranes; 2, membranes treated with 0.08 mM DTSSP for 15 min; 3, phosphorylase (M_r 92500), bovine serum albumin (M_r 66200), ovalbumin (M_r 45000), carbonic anhydrase (M_r 31000); 4, β-galactosidase (M_r 116000); 5, crosslinked bovine serum albumin. The positions of migration of the transhydrogenase subunits and of crosslinked subunits (A–C) are indicated.

We have previously found that the molecular weights of crosslinked species can be more accurately estimated from the rates of migration on SDS-PAGE if the Laemmli buffer system is replaced by phosphate buffer [7]. However, resolution between bands of similar molecular size is lost under these conditions. Fig. 3 shows gel electrophoresis of DTSSP crosslinked products in the phosphate buffer system. Bands A, B and C, equivalent to components 1, 2 and 3/4 of Fig. 2, migrated with apparent molecular weights of 211000,162000 and 106000. The α and β subunits migrated as a single band (M_r 52500). The molecular weights were obtained by comparison with the migration rates of standard proteins (Fig. 3). A plot of log M_r versus distance migrated by the protein gave an excellent linear plot (not shown).

Use of the data from Fig. 3 together with those of Fig. 2 confirmed that bands B and 2 were trimers $\alpha_2\beta$. Band A and 1 is a tetramer, likely $\alpha_2\beta_2$.

The results obtained for the membrane-bound enzyme were confirmed using soluble transhydrogenase purified to homogeneity by the method of Clarke and Bragg [5]. Crosslinking between suitably located cysteine residues was obtained using a procedure previously applied to the F_1 ATPase of *E. coli* to generate a specific crosslink between two subunits [10]. In this procedure the soluble transhydrogenase was passed through a small column of Sephadex G-50 in the absence of EDTA. The formation of disulfide crosslinks due to air oxidation was induced by traces of divalent cations present on the column. A one-dimensional gel of the soluble transhydrogenase after passage through the column gave four bands (1–4) of crosslinked subunits which were shown by two-dimensional electrophoresis (Fig. 4B) to be $\alpha_2\beta_2$, $\alpha_2\beta$, α_2 and $\alpha\beta$, respectively. A small amount of β_2 migrating in front of the $\alpha\beta$ band was also observed. Some α_2 was present prior to passage of the enzyme through the column (Fig. 4A). As in Fig. 2, there was a relatively large amount of irreversibly aggregated subunits of molecular weight 100000 migrating on the diagonal.

Radiation inactivation

Lyophilized membranes were irradiated with gamma radiation at 38°C for varying periods of time. The membranes were then resuspended by addition of an appropriate volume of water to reconstitute the original buffer of 50 mM Tris/5 mM $MgSO_4$/1 mM dithiothreitol/1 mM EDTA (adjusted to pH 7.8 with H_2SO_4).

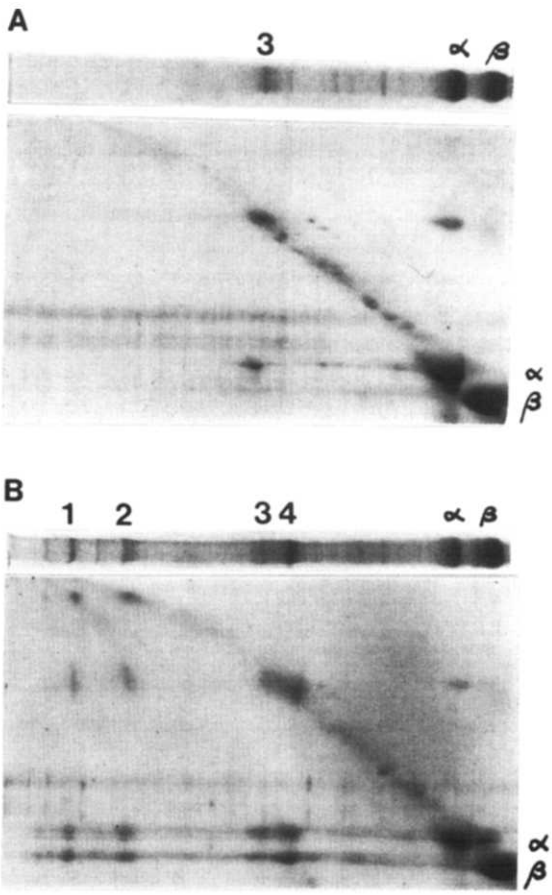

Fig. 4. Two-dimensional SDS-PAGE of crosslinked products arising from passage of purified solubilized transhydrogenase through a 1 ml column of Sephadex G-50 in 50 mM triethanolamine (adjusted to pH 7.5 with HCl). Disulfide crosslinks were formed by air oxidation in the presence of traces of divalent cations on the column. A, enzyme not passed through column; B, column-treated enzyme. The electrophoresis conditions are as in Fig. 2.

Samples were removed for analysis of energy-independent transhydrogenation of 3-acetylpyridine adenine dinucleotide by NADPH. The radiation inactivation size of 217 kDa, with a standard error of about 11%, was calculated from the equation given in Materials and Methods (Fig. 5).

Examination of the irradiated membranes by SDS-PAGE showed that the α subunit of the transhydrogenase was destroyed more rapidly than the β subunit as the radiation dose was increased. Scanning of the stained gels permitted calculation of the target sizes of the α and β subunits, where target size is defined as the mass of 1 mol of polypeptide(s) fragmented after a direct hit [8]. The log percentage of each subunit remaining was plotted versus radiation dose (Fig. 6). Target size was calculated from the equation given in Materials and Methods where $D_{37,t}$ is the radiation dose (in Mrad) necessary to destroy the subunit to 37% of its initial amount [8]. Target sizes of 98 ± 8 (six values) and 76 ± 8 (six values) kDa were calculated for the α and β subunits, respectively. These values are consistent with energy transfer between α_2 (M_r 107800) and β_2 (M_r 97300) oligomers of a tetrameric $\alpha_2\beta_2$ structure. The difference between target sizes of the α and β subunits may appear greater than in reality. When the larger α subunit is fragmented, peptides which would comigrate with the smaller size β subunit may increase the band intensity on the gel. This would in turn artifactually increase the target size calculated from apparent β band decay. It is not possible to evaluate the magnitude of the experimental error made but it is expected to be low considering the values obtained compared to the expected sizes. Therefore, it can be concluded that the destructive energy absorbed by a subunit following a hit is transferred exclusively to an identical subunit inside the oligomer causing the destruction of two subunits from a single hit.

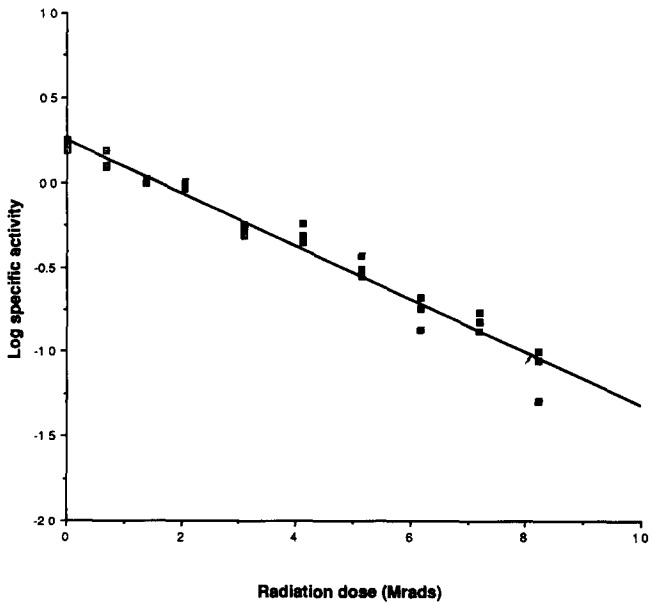

Fig. 5. Radiation inactivation curves for membrane-bound transhydrogenase. Radiation inactivation was carried out in triplicate as described in Materials and Methods. Each point is the mean of two or three assays.

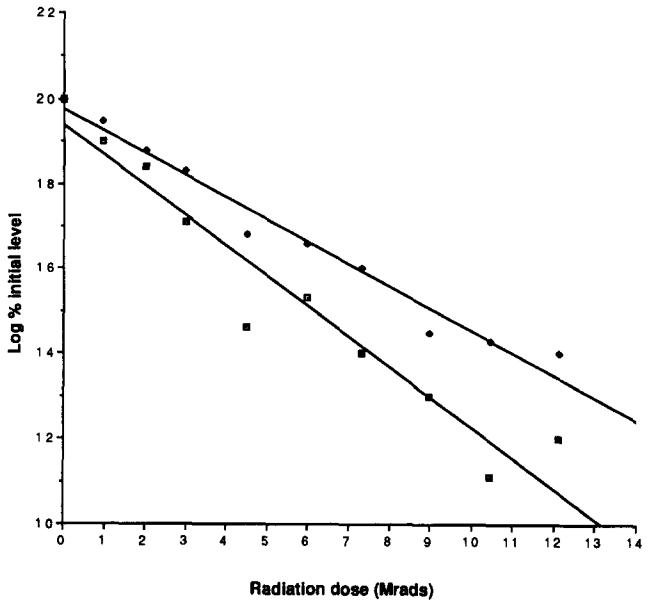

Fig 6. Determination of target size for α (open points) and β (closed points) subunits. Radiation inactivation was carried out as described in Materials and Methods. Samples after irradiation were run on SDS-polyacrylamide gels, stained with Coomassie blue and the intensity of the protein bands determined with a densitometer. The log of the amount of subunit remaining after irradiation is plotted versus amount of radiation received.

Discussion

Crosslinking of the transhydrogenase was most successfully accomplished by using cupric 1,10-phenanthrolinate to catalyse the formation of disulfide bridges between cysteine residues on different subunits. Some crosslinking within the same polypeptide chain was also observed. The advantage of using this crosslinking agent is that the presence of a crosslink between subunits is a clear indication that the two subunits are in close proximity in the molecule. It also offers the promise that identification of the sulfhydryl groups involved in the crosslink would permit determination of the sequences in contact in the two subunits.

The major product of crosslinking with both membrane-bound and solubilized enzyme was the α_2 dimer, but significant amounts of $\alpha\beta$ and β_2 dimers were formed. The trimer $\alpha_2\beta$ could be clearly identified. A tetramer, likely $\alpha_2\beta_2$, was also present in small amounts. The probability of three pairs of sulfhydryl groups being appropriately aligned to form the three disulfide bonds necessary to generate the tetramer is obviously lower than that of finding an appropriately aligned pair

for dimer formation. Crosslinking between α subunits occurs more readily than between β subunits whether the crosslinking agent is cupric 1,10-phenanthrolinate or is DSP or DTSSP. This is probably due to the topology of the transhydrogenase. Thus, studies with proteinases (Tong R. and Bragg, P.D., unpublished data) have suggested that the amino-terminal 80% of the α subunit projects from the membrane in everted membrane vesicles and would be more accessible to the crosslinker, whereas the β subunit, which was very much more resistant to proteolysis, must be shielded.

The tetrameric structure $\alpha_2\beta_2$ indicated by crosslinking was verified by radiation inactivation. Enzyme activity following irradiation was determined in the absence and presence of uncoupler, since we have found that the electrochemical gradient of protons generated during the reaction opposes further transhydrogenation. The radiation inactivation size of 217 kDa, determined in the presence of uncoupler, is consistent with the expected value (M_r, 205 146) for the $\alpha_2\beta_2$ structure and shows that the tetramer is the structure necessary for activity. Moreover, measurement of target size by radiation inactivation showed the destruction of two α or two β subunits from a single hit. Therefore, destruction of any pair of identical subunits in the tetramer causes inactivation of the whole oligomer. It is concluded that two pairs of subunits are required for the expression of transhydrogenation and that the tetramer likely represents the functional unit of the enzyme.

It is interesting to compare these results with those obtained with the mitochondrial transhydrogenase. The sequence of this enzyme has recently been determined [11]. The enzyme consists of a single polypeptide chain composed of sequences highly homologous with the α and β subunits of the *E. coli* transhydrogenase but separated by an additional 32 amino acids. Crosslinking [12,13] and radiation inactivation analysis [14] have suggested that the enzyme exists as a dimer, although the hydrodynamic properties of the purified transhydrogenase in 0.3% Triton X-100 are consistent with a monomeric structure [14]. However, the monomer was inactive.

In summary, there is good agreement between the results for the *E. coli* and mitochondrial transhydrogenases. The structural unit is the $\alpha_2\beta_2$ tetramer in *E. coli*. This is homologous to the dimeric protein of the mitochondrion. It is not entirely clear if the tetramer is the functional unit also. Two cooperatively interacting $\alpha\beta$ pairs are consistent with the data. Catalytic mechanisms for transhydrogenation based on cooperative interactions between subunits have been proposed [15,16].

Acknowledgement

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References

1 Rydström, J., Persson, B. and Carlenor, E. (1986) in Coenzymes and Cofactors (Dolphin, D., Poulson, R. and Avramovic, O., eds.), pp. 433–460, Wiley, New York.
2 Clarke, D.M., Loo, T.W., Gillam, S. and Bragg, P.D. (1986) Eur. J. Biochem. 158, 647–653.
3 Clarke, D.M. and Bragg, P.D. (1985) J. Bacteriol. 162, 367–373.
4 Clarke, D.M. and Bragg, P.D. (1986) FEBS Lett. 200, 23–26.
5 Clarke, D.M. and Bragg, P.D. (1985) Eur. J. Biochem. 149, 517–523.
6 Bragg, P.D. and Hou, C. (1980) Eur. J. Biochem. 106, 495–503.
7 Bragg, P.D. and Hou, C. (1986) Arch. Biochem. Biophys. 244, 361–372.
8 Beauregard, G., Maret, A., Salvayre, R. and Potier, M. (1987) Methods Biochem. Anal. 32, 313–343.
9 Kunkel, G.R., Mehrabian, M. and Martinson, H.G. (1981) Mol. Cell. Biochem. 34, 3–13.
10 Bragg, P.D. and Hou, C. (1986) Biochim. Biophys. Acta 851, 385–394.
11 Yamaguchi, M., Hatefi, Y., Trach, K. and Moch, J.A. (1988) J. Biol. Chem. 263, 2761–2767.
12 Anderson, W.M. and Fisher, R.R. (1981) Biochim. Biophys. Acta 635, 194–199.
13 Wu, L.N.Y. and Fisher, R.R. (1983) J. Biol. Chem. 258, 7847–7851.
14 Persson, B., Ahnström, G. and Rydström, J. (1987) Arch. Biochem. Biophys. 259, 341–349.
15 Enander, K. and Rydström, J. (1982) J. Biol. Chem. 257, 14760–14766.
16 Pennington, R.M. and Fisher, R.R. (1981) J. Biol. Chem. 256, 8963–8969.