Identification of Lysine 74 in the Pyruvate Binding Site of Alanine Dehydrogenase from Bacillus subtilis

CHEMICAL MODIFICATION WITH 2,4,6-TRINITROBENZENESULFONIC ACID, N-SUCCINIMIDYL 3-(2-PYRIDYLDITHIO)PROPIONATE, AND 5′-(p-(FLUOROSULFONYL)BENZOYL)ADENOSINE

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1-Alanine dehydrogenase from Bacillus subtilis was inactivated with two different lysine-directed chemical reagents, i.e. 2,4,6-trinitrobenzenesulfonic acid and N-succinimidyl 3-(2-pyridyldithio)propionate. In both cases, the inactivation followed pseudo-first-order kinetics, with a 1:1 stoichiometric ratio between the reagent and the enzyme subunits. Partial protection of the active site from inactivation could be achieved by each of the substrates, NADH or pyruvate, but complete protection could only be achieved in the presence of the ternary complex E-NADH-pyruvate. The nucleotide analogue of NADH, 5′-(p-(fluorosulfonyl)benzoyl)adenosine was also used for affinity labeling of the enzyme active site.

Differential peptide mapping, performed both in the presence and in the absence of the substrates, followed by reversed phase high performance liquid chromatography separation, diode-array analysis, mass spectrometry, and N-terminal sequencing of the resulting peptides, allowed the identification of lysine 74 in the active site of the enzyme. This residue, which is conserved among all L-alanine dehydrogenases, is most likely the residue previously postulated to be necessary for the binding of pyruvate in the active site.

Surprisingly, this residue and the surrounding conserved residues are not found in amino acid dehydrogenases like glutamate, leucine, phenylalanine, or valine dehydrogenases, suggesting that A-stereospecific amino acid dehydrogenases such as L-alanine dehydrogenase could have evolved apart from the B-stereospecific amino acid dehydrogenases.

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§ The abbreviations used are: AlaDH, alanine dehydrogenase; FSBA, 5′-(p-(fluorosulfonyl)benzoyl)adenosine; HPLC, high performance liquid chromatography; Rₜ, retention time; SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

oxidative deamination of L-alanine to pyruvate and ammonium (Reaction 1).

\[ \text{NAD}^+ + \text{H}_2\text{O} + L\text{-alanine} \rightarrow \text{NH}_4^+ + \text{pyruvate} + \text{NADH} + \text{H}^+ \]

**Reaction 1**

In *Bacillus* species this enzyme is known to play a key role in the generation of pyruvate as energy source during sporulation (5, 6). The kinetic properties of the enzyme have been elucidated (7), together with the mechanism (8) and the limiting steps of the catalysis (9). These studies showed that the *B. subtilis* alanine dehydrogenase follows predominately an ordered mechanism in which NAD⁺ binds before L-alanine. The products are released in the order ammonia and pyruvate before NADH. The hydrogen transfer to NAD⁺ during catalysis has been shown to occur at the pro(R) position of the nicotinamide ring, indicating that *B. subtilis* AlaDH is a member of the A-stereospecific dehydrogenases (10).

The amino acid sequence of AlaDH has been first determined from the strains *Bacillus sphaericus* and *Bacillus steatorrhophilus* (11). The sequence fingerprint characteristic of the βββ Rossmann’s fold responsible for the nucleotide binding (12, 13) has been recognized. Alignment of AlaDH sequences with other amino acid dehydrogenases like glutamate dehydrogenase, leucine dehydrogenase, or phenylalanine dehydrogenase, and with hydroxyacid dehydrogenases like lactate dehydrogenase or malate dehydrogenase led Kuroda et al. (11) to propose His-153 and Lys-156 to be part of the catalytic site. However, the availability of a third AlaDH sequence, obtained from *Mycobacterium tuberculosis* (14) did not support this hypothesis and stressed the need to investigate the composition of the active site (15).

Sequence comparisons between AlaDH and other proteins available in data banks have shown high similarities between AlaDH and the N-terminal part of pyridine nucleotide transhydrogenase (15, 16), for which a three-dimensional model of the NAD⁺ binding site has been proposed (17). However, no convincing sequence resemblance between AlaDH and the other enzymes of the amino acid dehydrogenase superfamily has been found. In that respect, AlaDH constitutes an exception from the other amino acid dehydrogenases, like glutamate dehydrogenase, whose three-dimensional structure has been elucidated (18), or leucine dehydrogenase, phenylalanine dehydrogenase, and valine dehydrogenase, which have been shown to share sequence and structure similarities with glutamate dehydrogenase (19, 20). These similarities include an identical B-type stereospecificity with respect to NAD⁺ (21–23) and a common organization of the residues implicated in the catalytic chemistry (19).

We have much information on the active-site structure of...
B-stereospecific amino acid dehydrogenases, obtained by chemical modification studies (24–28), by genetic engineering (27, 29–32), and from x-ray crystallographic data (18, 33, 34). However, very scarce information is available for the A-stereospecific amino acid dehydrogenases such as alanine dehydrogenase. Little is known about the residues that might be involved in substrate binding and catalysis (8, 35, 36), and no catalytic amino acid residue has been identified. Recently, the amino acid sequence from B. subtilis alanine dehydrogenase has become available (6), allowing the interpretation of chemical modification studies performed on this enzyme.

In this paper, we provide evidence that Lys-74 of B. subtilis alanine dehydrogenase is located at the active site of the enzyme. That residue is conserved among all alanine dehydrogenases sequenced so far and is likely the lysine residue that is required for the binding of pyruvate during the catalytic reaction.

EXPERIMENTAL PROCEDURES

Materials—L-Alanine dehydrogenase from B. subtilis purchased from Sigma was desalted before use on a 15 × 2.5-cm Ultragel AcA44 column (IBF Biotechnics, Villeneuve-la-Garenne, France) equilibrated in 100 mM NaH2PO4, pH 7.5. The substrates L-AlaDH and pyruvate and the chemical reagents 2,4,6-trinitrobenzenesulphonate acid (TNBS), N-succinimidyl 3-2-pyridyldithio)propionate (SPDP), and 5′-(fluorosulfonyl)benzoyl)adenosine (FSBA) were also obtained from Sigma. Endoproteinase Glu-C sequencing grade was from Boehringer (Mannheim, Germany). All other reagents were from Merck (Darmstadt, Germany), except Tris, guanidine HCl, and trifluoroacetic acid, which were from Acros Chimica (Beerse, Belgium), and acetonitrile, which was from Carlo Erba (Milano, Italy).

Assays—Alanine dehydrogenase activity was assayed for alanine synthesis according to Yoshida et al. (3). In typical experiments, the assay mixture (0.6 ml) contained 0.5 mM NADH, 2 mM pyruvate, and 100 mM NH4Cl in 100 mM Tris-HCl, pH 8.5. The reaction was started by the addition of 1–2 μg of protein to the mixture. The assay was carried out at 20 °C by recording the decrease in absorbance of NADH at 340 nm, with a Kontron 930 variable wavelength spectrophotometer. Protein assay was performed by the Folin reagent method (37), using bovine serum albumin as standard. When the samples contained Tris-HCl as buffer, the protein concentration was determined by the dye binding assay (38), using the kit provided by Bio-Rad (München, Germany).

Chemical Modification with TNBS—L-AlaDH was inactivated by incubating the enzyme (1 mg/ml) in the dark, at 40 °C, in the presence of varying concentrations of TNBS (39) (stock solutions diluted in ice-cold distilled water), in 100 mM NaH2PO4, pH 8.0. Aliquots (2 μl) were removed at regular time intervals and mixed with the assay solution to measure residual activity. Control experiment showed that the enzyme did not lose any activity in those conditions when TNBS was omitted. When necessary, peptides of interest were further purified by HPLC under the same chromatographic conditions, except that a linear gradient of 0.25% increase of solvent B/min was used. Alternatively, peptides were purified by HPLC on a C18 column from Pharmacia (Uppsala, Sweden) using the Pharmacia Smart system with a linear gradient of 0–70% solvent B over 45 min.

Mass Analysis of Peptides and Amino Acid Sequence Determination—Peptides of interest were dissolved in acetonitrile/1% acetic acid in water (50:50) and their mass determined in a VG Platform electrospray ionization mass spectrometer (Fisons Instruments, Manchester, United Kingdom). The amino acid sequence of peptides was determined on a model 475A peptide sequencer (Applied Biosystems, Foster City, CA) equipped with on-line phenylthiohydantoin-derivative analyzer.

RESULTS

Chemical Modification by TNBS and SPDP—TNBS and N-hydroxysuccinimide esters like SPDP are known to be highly selective reagents at pH 7.5–8.0 for the modification of lysine residues (ε-NH2 group of the side chain) and/or N terminus of proteins (39, 40). Incubation of L-alanine dehydrogenase with varying concentrations of TNBS and SPDP resulted in a time-dependent loss of enzyme activity, suggesting the modification of a primary amino group located at or near the enzyme active site. Plots of the logarithm of remaining activity versus time at different reagent concentrations indicated in each case pseudo first-order kinetics (Figs. 1A and 2A). A straight line was also observed for the plot of pseudo first-order rate constants versus reagent concentration (Figs. 1B and 2B), indicating that the chemical modification is the result of a simple bimolecular reaction. The second-order rate constants (kmax) obtained for the modification by TNBS and SPDP were 0.88 mmol/min/mg (60 min) and 0.44 mmol/min/mg (10 min), respectively, and show a much higher reactivity of SPDP compared to TNBS toward the active-site lysine residue. Plotting log kmax versus log of reagent concentration, according to Levy et al. (43), yields an apparent reaction order of 0.88 and 1.00 for TNBS and SPDP, respectively, indicating that inactivation results from the reaction of approximately 1 mol of
enzyme (hexamer) concentration. Samples (2 × 10³ l) were removed every 5 min, and residual activity was determined. Pseudo first-order rate constants of inactivation \(k_{\text{inact}}\) are obtained from the slopes of straight lines fitted to data points by least square linear regression. The activity declined to a non-zero value after a relatively long period (60 min of incubation). A minimum residual activity of about 25% was obtained with 10 mM FSBA (Fig. 5A). The rate of inactivation increased according to the initial FSBA concentration, showing typical saturation kinetics. This is observed in the plot of pseudo first-order rate constants \(k_{\text{inact}}\) versus FSBA concentration (Fig. 4A) and in the plot of \(\log k_{\text{inact}}\) versus \(\log \text{FSBA concentration}\) (Fig. 4C), where a deviation from linearity is observed at high reagent concentration. The activity declined to a non-zero value after a relatively long period (60 min of incubation). A minimum residual activity of about 25% was obtained with 10 mM FSBA (Fig. 5A). The rate of inactivation increased according to the initial FSBA concentration, showing typical saturation kinetics. This is observed in the plot of pseudo first-order rate constants \(k_{\text{inact}}\) versus FSBA concentration (Fig. 4A) and in the plot of \(\log k_{\text{inact}}\) versus \(\log \text{FSBA concentration}\) (Fig. 4C), where a deviation from linearity is observed at high reagent concentrations. Fitting straight lines by linear regression on the linear portion of data points gave values of 5.47 × 10⁻² M⁻¹ s⁻¹ and 0.76 for the second-order rate constant and the apparent order of reaction, respectively, but a polynomial second-order curve best fitted the data points in Fig. 4B with a correlation coefficient of 0.97. These results clearly show that chemical modification of AlaDH with FSBA does not follow a simple bimolecular mechanism as observed for TNBS or SPDP but proceeds through a two-step reaction. This is in agreement with a mechanism where FSBA first binds to the adenosine binding site of the enzyme to form a reversible non-covalent complex (inhibition) and subsequently reacts in an irreversible covalent way with an amino acid residue of the active site (inactivation) (Reaction 2).

\[
E + \text{FSBA} \rightleftharpoons \text{E·FSBA} \rightleftharpoons \text{E-FSBA}
\]

\(k_1\) non-covalent complex (inhibition) \(k_2\) covalent complex (inactivation)

**Fig. 1.** Inactivation of alanine dehydrogenase with TNBS. A, L-alanine dehydrogenase (1 mg/ml, 4.4 × 10⁻³ M, 60 µl) in 100 mM NaH₂PO₄, pH 7.5 (20 °C), was incubated with no (●), 20 × (○), 50 × (▲), and 200 × (△) molar excess of reagent relative to the enzyme (hexamer) concentration. Samples (2 × 10³ l) were removed every 5 min, and residual activity was determined. The activity declined to a non-zero value after a relatively long period (60 min of incubation). A minimum residual activity of about 25% was obtained with 10 mM FSBA (Fig. 5A). The rate of inactivation increased according to the initial FSBA concentration, showing typical saturation kinetics. This is observed in the plot of pseudo first-order rate constants \(k_{\text{inact}}\) versus FSBA concentration (Fig. 4A) and in the plot of \(\log k_{\text{inact}}\) versus \(\log \text{FSBA concentration}\) (Fig. 4C), where a deviation from linearity is observed at high reagent concentrations. Fitting straight lines by linear regression on the linear portion of data points gave values of 5.47 × 10⁻² M⁻¹ s⁻¹ and 0.76 for the second-order rate constant and the apparent order of reaction, respectively, but a polynomial second-order curve best fitted the data points in Fig. 4B with a correlation coefficient of 0.97. These results clearly show that chemical modification of AlaDH with FSBA does not follow a simple bimolecular mechanism as observed for TNBS or SPDP but proceeds through a two-step reaction. This is in agreement with a mechanism where FSBA first binds to the adenosine binding site of the enzyme to form a reversible non-covalent complex (inhibition) and subsequently reacts in an irreversible covalent way with an amino acid residue of the active site (inactivation) (Reaction 2).

**Fig. 2.** Inactivation of alanine dehydrogenase with SPDP. A, L-alanine dehydrogenase (1 mg/ml, 4.4 × 10⁻³ M, 60 µl) in 100 mM NaH₂PO₄, pH 7.5 (20 °C), was incubated with no (●), 20 × (○), 50 × (▲), 10 × (△), 25 × (□), and 50 × (△) molar excess of reagent relative to the enzyme (hexamer) concentration. Samples (2 µl) were removed every minute, and residual activity was determined. See Fig. 1 legend for details.
For this reason, results were analyzed according to the method of Kitz and Wilson (44) for irreversible inhibitors, where the observed rate constant for inactivation is as shown in Equation 1.

\[
k_{\text{inact}} = \frac{[\text{FSBA}]}{[\text{FSBA}] + K_f} + \frac{1}{k_3}
\]  
(Eq. 1)

The double-reciprocal plot according to this method, \(1/k_{\text{inact}}\) \(v\)ersus \(1/[\text{FSBA}]\), gives a straight line (Fig. 5B).

For Protection Studies—In order to assess that the chemical modification by TNBS, SPDP, and FSBA is active-site-directed, we tested the ability of substrates to protect the enzyme active site from inactivation (Fig. 6). The mechanism of reaction of AlaDH is known to be ordered with first the binding of NADH, followed by pyruvate and ammonium (7). We could not assay the protection by \(l\)-alanine or NH\(_4\)Cl since they can react with the chemical reagents used. We tested the protecting effect of NADH, pyruvate, and pyruvate analogues. In the case of TNBS and SPDP, only little or no protecting effect was observed when the substrates NADH or pyruvate were used alone. However, the \(k_{\text{inact}}\) was dramatically reduced when NADH and pyruvate were used together. This result clearly shows that the lysine residue reacting with these chemicals is located in the active site of the enzyme.
Protection of alanine dehydrogenase by substrates and analogues during inactivation with SPDP

Table I

| Protecting substrates and analogues | Structure HOOC-CO-R | k_{inact} | Residual activity | Protecting effect | K_{i}^{a} |
|-----------------------------------|---------------------|-----------|------------------|------------------|----------|
|                                   | 10^{-2} min^{-1}    | %         | %                |                  | nM      |
| No protection                     | 10.31               | 12.1      | <0               | 0.53             |         |
| Pyruvate                          | 8.86                | 15.2      | <0               | 0.53             |         |
| NADH                             | 2.83                | 53.7      | 0                | 2.3 × 10^{-2}    |         |
| NADH + glyoxylate                 | R = -H              | 3.23      | 20.6             | <0               | 16      |
| NADH + pyruvate                   | R = -CH_{3}         | 1.66      | 71.5             | 50               | 23      |
| NADH + α-ketobutyrate             | R = -(CH_{2})_{2}-CH_{3} | 2.68 | 58.3             | 6               | 33      |
| NADH + oxalate                    | R = -OH             | 2.77      | 54.7             | 2                |         |
| NADH + mesoxalate                 | R = -COOH           | 3.24      | 49.6             | <0               |         |
| NADH + oxalacetate                | R = -(CH_{2})_{2}-COOH | 1.57 | 70.2             | 53               | 6.5^{c} |
| NADH + α-ketoglutarate            | R = -(CH_{2})_{3}-COOH | 3.14 | 51.3             | <0               |         |
| NADH + oxamate                    | R = -NH_{2}         | 2.04      | 67.7             | 33               |         |

a The protecting effect is calculated in percent relative to the protective effect of NADH + pyruvate taking the protecting effect of NADH alone as being zero.
b In 5 mM Tris-HCl, pH 8.0 (25 °C), according to Ref. 3.
c Determined by ourselves in 100 mM Tris-HCl, pH 8.5 (20 °C).
d Inhibition constant for oxamate in 50 mM Tes, pH 7.9 (25 °C), K_{i} = 4.1 mM, according to Ref. 7.

Identification of Modified Amino Acid Residues—L-Alanine dehydrogenase was chemically modified using TNBS, SPDP, and FSBA as described under “Experimental Procedures.” Samples were passed through a desalting column, reconcentrated, and submitted to proteolysis using endoproteinase Glu-C (42). For TNBS and SPDP inactivation, experiments were carried out in parallel both in the presence of NADH and pyruvate (protection of the whole active site) or in the presence of NADH alone (protection of the NADH binding site only).

The HPLC profiles obtained for TNBS-modified alanine dehydrogenase are presented in Fig. 7. The comparison of the profiles shows that the peak eluting at 32.5 min disappears when pyruvate is absent from the active site (Fig. 7, A and B, peak I), while another peak eluting at 46.0 min increases (peak II). This increase is particularly obvious at 346 nm (Fig. 7D), which is a specific wavelength for TNBS chemical modification. Diode-array detection analysis of these compounds indicated that peak II was labeled with TNBS, while peak I was not, as shown by its characteristic absorbance profile with a maximum at 346 and 420 nm (Fig. 8A). Peptides from peaks I and II were collected and submitted to mass determination and to N-terminal sequencing (over 6–10 residues) by automated Edman degradation. For the peak eluting at 32.5 min, the sequence MVMKVK could be identified (Table II). This result, together with the mass determination obtained (M_{r} = 2286.8) indicates that peptide I corresponds to Met-69 to Lys-86 (Table III).

Interestingly, a similar sequence was found in the peptic fragments identified in peak II, where the mass observed for one of these two fragments was consistent with the TNBS labeling of the peptide Met-69 to Lys-86 (Table II, fragment Ia). Unfortunately, only a few phenylthiohydantoin-derivatives of this fragment could be identified due to the low amount of material available, but they all corresponded to the expected sequence. Another peptic fragment (Table II, fragment IIa), starting with a proline corresponding to Pro-76 in the AlaDH sequence, contaminated peak II.

The HPLC profile of the peptides obtained after chemical modification of alanine dehydrogenase with SPDP is presented in Fig. 9. Similarly to the results obtained in Fig. 7, the peak eluting at R_{t} = 32.5 min (peak I) decreases when pyruvate is absent from the active site, while another peak increases, which absorbs at 304 nm (Fig. 8B) and elutes at R_{t} = 39.8 min (Fig. 9, peak II). This result suggests that the SPDP chemical labeling modified the same peptic fragment of alanine dehy-
B-Phe-containing peptides. Characteristic of SPDP labeling (40); characteristic of FSBA labeling (41).

This was also confirmed by the mass analysis and sequence determination of the peptides contained in peak III (Table II),

MVMKV*EP, with an unidentified phenylthiohydantoin-derivative at the 6th cycle, corresponding to Lys-74 in L-alanine dehydrogenase (Table II).

The elution pattern of the peptides resulting from endopeptidase Glu-C digest of FSBA-labeled alanine dehydrogenase (Fig. 10) was obtained similarly to those obtained after the TNBS and SPDP chemical modification, except that the experiment was performed both in the presence of NADH and pyruvate (protection of the whole active site) and without any substrate (no protection). This reagent, which potentially can react with several residues of the active site, also reacted with the same peptide fragment as indicated by the decrease of peak I when the substrates were omitted (Fig. 10, A and B). Peak IV appearing in those conditions highly absorbs at 259 nm (Fig. $8C$ and $10D$) and gave the sequence MVMKV*EPLP, with masses of 2721.0 and 3838.0 (Table II).

### Table II

| Sample (peak) | Chemical modification | N-terminal sequence | Masses observed |
|---------------|-----------------------|---------------------|----------------|
| I             | None                  | MVMKVK              | 2286.8         |
| IIa           | TNBS                  | XXVXV*PLPEEYV       | 2498.5         |
| IIb           | TNBS                  | PLPEEYYYFR          | 3374.5         |
| III           | SPDP                  | MVMKV*EP            | 2374.2, 2484.8, and 3912.1 |
| IV            | FSBA                  | MVMKVEPLP           | 2721.0 and 3883.0 |

where masses of 2484.8 and 2374.2 are in agreement with the SPDP-labeling of peptide I (in the oxidized and reduced states), while the mass of 3912.1 is consistent with a longer SPDP-labeled fragment from Met-69 to Glu-99. The N-terminal sequencing of the peptide from peak III gave the consensus sequence MVMKV*EP, with an unidentified phenylthiohydantoin-derivative at the 6th cycle, corresponding to Lys-74 in L-alanine dehydrogenase (Table II).

### DISCUSSION

L-Alanine dehydrogenase from *B. subtilis* was completely inactivated using TNBS and SPDP. In both cases, the inactivation of the enzyme was the result of a simple bimolecular reaction, with the modification of about one lysine residue/monomer. This result suggests the presence of an essential lysine residue at or near the active site of the enzyme. A different pattern of inactivation was obtained for the modification of AlaDH with the structural analogue of NADH, *i.e.* FSBA. In this case, saturation kinetics were obtained, indicating a stepwise mechanism where FSBA binds to the NADH binding site of the enzyme before irreversible chemical modification of an active-site residue. Results obtained for the chemical modification of the enzyme in the presence of the substrates indicate that NADH or pyruvate alone do not allow a good protection, and that the enzyme can only be effectively protected when the ternary complex E/NADH/pyruvate is formed. Given the ordered mechanism of AlaDH where NADH and pyruvate is required for a good protection, and that the enzyme can only be effectively protected when the ternary complex E/NADH/pyruvate is formed. Given the ordered mechanism of AlaDH where NADH and pyruvate are required for a good protection of the active site sug-

Differential peptide mapping, both in the presence and in the absence of the substrates, and monitoring at a wavelength specific for the label, allowed the identification of active-site peptide fragments. For both TNBS and SPDP, the same peptidic fragment from Met-69 to Lys-86 was found to be labeled, with a concomitant retention time modification and increase of
Table III

Alignment of the active-site peptide fragment with the sequences of L-alanine dehydrogenase and pyridine nucleotide transhydrogenase

The asterisk indicates the lysine residue modified during the chemical labeling of the L-alanine dehydrogenase active site. Outlined letters denote residues conserved among all alanine dehydrogenases sequenced to date. The number of the first and last residue of each sequence is given, respectively, at the beginning and end of each line. Residues EEGTD, PTLGVH, PTLGAH, and PTLAVH, are, respectively, in the original sequences of Rhodospirillum rubrum, Bos taurus, Mus musculus, and Homo sapiens pyridine nucleotide transhydrogenases before the conserved glutamate residue.

| Sequence Origin | Active site peptide | This paper |
|-----------------|---------------------|------------|
| B. subtilis AlaDH | AEMV MKEKLPE YV FRK | Siranosian et al., 1993 (6) |
| B. sphaericus AlaDH | QEMLKKEKLPE YV FRK | Kuroda et al., 1990 (11) |
| B. stearothermophilus AlaDH | AEMV MKEKLPE YV FRK | Kuroda et al., 1990 (11) |
| M. tuberculosis AlaDH | ADLKLKEKIAEYGLRLH | Andersen et al., 1992 (14) |
| Synechocystis sp AlaDH | RELV VKEKLPE YEYLTLPK | Kaneko et al., 1996 (48) |
| Synechocystis sp PNT | ADIlUKRPSAEEVLPAKG | Kaneko et al., 1996 (48) |
| Escherichia coli PNT | SEILKNAPLDDLTAIALLNP | Ahmad et al., 1992 (49) |
| Haemophilus influenzae PNT | SDIIFNASPDTEELAQMEKGA | Fleischmann et al., 1995 (50) |
| Rhodospirillum rubrum PNT | ADV WRQPMTEDEVLKKEGA | Williams et al., 1994 (51) |
| Entamoeba histolytica PNT | SNICKNPSPSEELINKMROQG | Yu & Samuelson, 1994 (52) |
| Caenorhabditis elegans PNT | TDILKRPSTENYKLSGC | Wilson et al., 1994 (53) |
| Bos taurus PNT | SDLVKKRAPMLNADLKLTS | Yamaguchi et al., 1988 (54) |
| Mus musculus PNT | SDLVKKRAPMNADFLKPS | Arkblad et al., 1996 (55) |
| Homo sapiens PNT | SDLVKKRAPMNADLKLTS | Arkblad et al., 1996 (55) |

Fig. 9. Reversed phase HPLC profile of the peptides resulting from the endoproteinase Glu-C proteolysis of SPDP-modified L-alanine dehydrogenase. A and C, chemical modification in the presence of NADH and pyruvate (protection of the whole active site); B and D, chemical modification in the presence of NADH alone (protection of the NADH binding site only). Arrows indicate active-site peptides. Mass determination: peptides from peak III, $M_r = 2374.2, 2484.8$, and 3912.1.

Fig. 10. Reversed phase HPLC profile of the peptides resulting from the endoproteinase Glu-C proteolysis of FSBA-modified L-alanine dehydrogenase. A and C, chemical modification in the presence of NADH and pyruvate (protection of the active site); B and D, chemical modification without substrates (no protection). Arrows indicate active-site peptides. Mass determination: peptides from peak IV, $M_r = 2721.0$ and 3883.0.

absorbance of the corresponding peak. Sequence comparisons of the isolated fragments with the known sequence of B. subtilis L-alanine dehydrogenase (6) indicated that the cleavage between Glu-68 and Met-69 is consistent with the expected cleavage specificity of endoproteinase Glu-C, i.e. the cleavage after Glu or Asp residues (42). On the other hand, the absence of cleavage after Glu-75, Glu-79, and Glu-80 is probably due to the presence of two proline residues, respectively, at position 76 and 78 of the sequence. These residues are known to form secondary structures that often prevent the recognition of pro-
teases with their substrates. More surprising was the aspecific cleavage of endoprotease Glu-C after a lysine residue in position 86, which was obtained for several independent experiments. This unusual cleavage after a lysine residue is not in the list of the several aspecific cleavages that have been reported for endoprotease Glu-C, for example after Gly and Ala (45), Asn and Tyr (46), or Gln and Ser (47). To our knowledge, cleavage after a lysine had not yet been observed.

Mass and sequence determinations of the isolated peptides were consistent with the chemical labeling of lysine 74 of the l-alanine dehydrogenase, implying the presence of this residue at or near the active site of the enzyme. Interestingly, the active-site affinity labeling using FSBA also modified the same Met-69--Lys-86 peptic fragment at the position of lysine 74, although FSBA can react with several residues other than lysine. Sequence alignment of this peptide with the known sequences of B. subtilis AlaDH (6), B. sphaericus (11), B. stearo-
thermophilus (11), M. tuberculosis (14), and Synechocystis sp. (48) indicates that the modified lysine residue is conserved among all the alanine dehydrogenases sequenced to date, and that it is located in an important stretch of five conserved residues KVKEP from Lys-72 to Pro-76 (Table III). According to the sequence analysis of the enzyme (15), these residues are most likely located outside of the NADH binding site, which is supposed to expand around and after the characteristic GXGXXG(X_2)D motif of the \(\beta\alpha\beta\) Rossmann’s fold. This observation is in agreement with a localization of Lys-74 at the pyruvate binding site but in close vicinity of the NADH binding site, since the same Lys-74 is also modified by FSBA. The presence of Lys-74 in the NADH binding site itself is not completely excluded. In this case, the protection obtained for the enzyme inactivation by TNBS and SPDP, only when both NADH and pyruvate are present, would implicate that Lys-74 would be part of the NADH binding site. This possibility cannot be ruled out, but all the arguments presented here above argue against this explanation.

According to Grimshaw et al. (8), a cationic acid group on the enzyme (probably a lysine) is required for effective binding of the substrate and the inhibitors, while another cationic acid group (probably a histidine), acts as an acid-base catalyst of the reaction. In an attempt to locate these residues in the sequence of B. sphaericus and B. stearothermophilus, Kuroda et al. (11) performed sequence comparisons with other amino acid dehydrogenases (glutamate dehydrogenase, phenylalanine dehydrogenase, and leucine dehydrogenase) and with hydroxycald dehydrogenase, which share some substrate and catalytic features with alanine dehydrogenase (lactate dehydrogenase and malate dehydrogenase). In their conclusions, the authors proposed His-153 and Lys-156 from B. sphaericus to be part of the active site (11, 27). However, the availability of a third alanine dehydrogenase obtained from M. tuberculosis (14) ruled out this hypothesis, since the proposed residues were not conserved in this new sequence (15). The experimental results obtained by us clearly identify Lys-74 as part of the enzyme active site, and support its role in the catalytic mechanism of the l-alanine dehydrogenase.

In a previous paper the sequence of B. sphaericus alanine dehydrogenase was compared with the protein sequences of the Swisssprot, GenBank, and EMBL data bases (15). Surprisingly, no other amino acid dehydrogenase or hydroxycald dehydrogenase was found to be significantly similar to alanine dehydrogenase, but the enzyme was found to be similar to the N-terminal sequence of pyridine nucleotide transhydrogenase, suggesting a similar folding of these two protein segments (15). However, no alanine dehydrogenase activity was detected in M. tuberculosis pyridine nucleotide transhydrogenase (56), suggesting that even if they have a similar structure, their active site is different. In agreement with this active-site difference, pyridine nucleotide transhydrogenase also lacks lysine 74, which was found to be essential for the activity of alanine dehydrogenase (Table III).

1-Alanine dehydrogenases appear as very unique enzymes among the amino acid dehydrogenases. Alanine dehydrogenase has been shown to be a member of \(\alpha\)-sterespecific dehydrogenases (10, 57), unlike the other amino acid dehydrogenases studied to date, which are \(\beta\)-stereospecific (21–23). In this paper, we showed that alanine dehydrogenases from B. subtilis possess a lysine at the position 74 that is essential for the enzyme activity, and that this residue is conserved among the other alanine dehydrogenases sequenced so far (Table III). This lysine and the surrounding conserved sequence region are not found in other dehydrogenases. Furthermore, the characteristic active-site motif K\(\beta\alpha\beta\) GGXK identified in glutamate, leucine, phenylalanine, and valine dehydrogenases (19, 20) is not found in l-alanine dehydrogenases, suggesting a separate evolution of these two groups of amino acid dehydrogenases. The only similarity of sequence was found with one part of the pyridine nucleotide transhydrogenase, which suggests that l-alanine dehydrogenase, contrary to the \(\beta\)-stereospecific amino acid dehydrogenases, may have evolved along with pyridine nucleotide transhydrogenases rather than with other dehydrogenases of the amino acid dehydrogenase superfamily.

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