Amino Acid Sequence of the Nucleotide-Binding Site of D-(−)-β-Hydroxybutyrate Dehydrogenase Labeled with Arylazido-β-[3-^{3}H]alanylnicotinamide Adenine Dinucleotide†

Mutsuo Yamaguchi,‡ Shiuan Chen,§ and Youssef Hatefi*,‡

Division of Biochemistry, Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California 92037, and Division of Immunology, Beckman Research Institute of City of Hope, Duarte, California 91010

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ABSTRACT: In the dark, arylazido-β-alanylnicotinamide adenine dinucleotide (N_3-NAD) can replace NAD as cofactor for D-(−)-β-hydroxybutyrate dehydrogenase (BDH) purified from bovine heart mitochondria. When photoirradiated with visible light, N_3-NAD forms a nitrene species that binds covalently to BDH and inhibits the enzyme. NAD(H) protects BDH against photolabeling and inhibition by N_3-NAD [Yamaguchi, M., Chen, S., & Hatefi, Y. (1985) *Biochemistry 24*, 4912–4916]. In the present study, a tryptic peptide of purified BDH photolabeled with arylazido-β-[3-^{3}H]alanyl-NAD ([^{3}H]N_3-NAD) was isolated and sequenced. The same tryptic peptide was also isolated from BDH not labeled with [^{3}H]N_3-NAD and sequenced. Both peptides indicated the sequence Met-Glu-Ser-Tyr-Cys-Thr-Ser-Gly-Ser-Thr-Asp-Thr-Ser-Pro-Val-Ile-Lys. The residue labeled with [^{3}H]N_3-NAD was Cys. This heptadecapeptide contains 14 uncharged residues and is marked by having in an undecapeptide segment 8 hydroxy amino acids located symmetrically around a central glycine.

D-(−)-β-Hydroxybutyrate dehydrogenase (BDH)[1] is a phospholipid-requiring enzyme that is associated with the mitochondrial inner membrane. BDH catalyzes the reversible oxidation of D-(−)-β-hydroxybutyrate to acetoacetate in the presence of NAD as the coenzyme. Similarly to LDH and MDH, bovine mitochondrial BDH appears to contain essential arginyl (Phelps & Hatefi, 1981b; Fleer & Fleischer, 1983), carboxyl, and histidyl (Prasad & Hatefi, 1986) residues at the active site. In addition, like LDH and mitochondrial MDH (Holbrook et al., 1975; Banaszak & Bradshaw, 1975), BDH contains an essential thiol (Phelps & Hatefi, 1981a; Fleer et al., 1984). The photosensitive NAD analogue arylazido-β-alanyl-NAD (N_3-NAD) can replace NAD in the dark as a coenzyme for BDH. The apparent K_m for N_3-NAD is only about twice that of NAD, but the turnover number of BDH in the presence of N_3-NAD is less than one-tenth the turnover number in the presence of NAD (Yamaguchi et al., 1985). Irradiation of BDH + N_3-NAD with visible light results in inhibition of the enzyme and labeling of the protein when [^{3}H]N_3-NAD is used. The inhibition and labeling are prevented by NADH, NAD + β-hydroxybutyrate, or NAD + 2-methylmalonate (Yamaguchi et al., 1985). These results indicated that the nitrene species produced from photoactivation of N_3-NAD inserts into the NAD(H) binding site of BDH. This paper describes the sequence of a tryptic peptide isolated from purified BDH, which had been photolabeled with [^{3}H]N_3-NAD. The isolated peptide is composed of 17 amino acid residues, is rich in serine and threonine, and contains one cysteine residue, which appeared to be the site of labeling with [^{3}H]N_3-NAD.

Materials and Methods

The sources of materials used were as follows: TPCK-treated trypsin was from Worthington; trifluoroacetic acid was from Aldrich; NAD was from Chemical Dynamics; acetonitrile (HPLC grade), methanol (HPLC grade), and chloroform were from Baker; DL-β-hydroxybutyrate, β-mercaptoethanol, and sodium iodoacetate were from Sigma; asolectin was from Associated Concentrates; dithiothreitol was from Calbiochem-Behring; phenylboronate–agarose (PBA-30 form) was from Amicon. Arylazido-β-alanyl-NAD and arylazido-β-[3-^{3}H]alanyl-NAD were synthesized according to Chen and Guillory (1977).

Preparation and Assay of BDH. Apo-BDH was purified from bovine heart mitochondria and assayed for activity as before (Yamaguchi et al., 1985). Protein concentration was determined by the method of Lowry et al. (1951) as modified by Peterson (1977), using bovine serum albumin as standard.

Photoaffinity Labeling of BDH. Purified apo-BDH (1.5 mg in 2.0 mL of 10 mM Tris–acetate, pH 8.0, containing 1 mM EDTA) was reconstituted with asolectin (150 μg of phospholipid phosphorus/mg apo-BDH) by incubation for 20 min at 37 °C and then passed through a Sephadex G-50 column (1.0 × 12 cm) equilibrated with 10 mM potassium phosphate, pH 7.3, containing 1 mM EDTA. To the eluate was added 0.1 mM [^{3}H]N_3-NAD (1.5×10^6 cpm/μmol), and the mixture was photoirradiated for 4 min under the conditions described previously (Yamaguchi et al., 1985). Eighty percent of the enzyme activity was lost by this treatment. The mixture was then passed through a second Sephadex G-50 column (1.0 × 12 cm), equilibrated with 10 mM ammonium bicarbonate, and lyophilized. Phospholipid concentration was estimated by phosphorus analysis according to Baginski et al. (1967).

Tryptic Digestion of BDH. The phospholipid-reconstituted BDH has been shown to be resistant to tryptic digestion (Berrez et al., 1984; Maurer et al., 1985). Therefore, phospholipid was extracted from the lyophilized, [^{3}H]N_3-NAD-

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* Author to whom correspondence should be addressed.
‡ Scripps Clinic and Research Foundation.
§ Beckman Research Institute of City of Hope.

[1] Abbreviations: BDH, LDH, MDH and GAPDH, D-(−)-β-hydroxybutyrate, lactate, malate, and glyceraldehyde-3-phosphate dehydrogenases, respectively; NAD, nicotinamide adenine dinucleotide; N_3-NAD, arylazido-β-alanyl-NAD; [^{3}H]N_3-NAD, arylazido-β-[3-^{3}H]-alanyl-NAD; FPLC, fast performance liquid chromatography; HPLC, high-performance liquid chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Cm, carboxymethyl.

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treated BDH by addition of 3 mL of chloroform/methanol (2:1). The mixture was stirred well for several minutes and then centrifuged in a clinical centrifuge at top speed. The supernatant layer was discarded, and the precipitated protein was taken up in 0.8 mL of 0.5 M Tris-HCl, pH 8.0, containing 6 M guanidine hydrochloride and 2 mM EDTA. The solution was treated sequentially with dithiothreitol and sodium iodoacetate and finally with β-mercaptoethanol to quench excess iodoacetate. The sample was dialyzed against distilled water and then further dialyzed against 10 mM ammonium bicarbonate overnight. The solution was then treated with TPCK–trypsin (trypsin to BDH ratio of approximately 1:50) and incubated at room temperature for 6 h. A sample of BDH not subjected to photoaffinity labeling with [^{3}H]N_3-NAD was similarly treated, carboxymethylated, and digested with TPCK–trypsin.

Chromatography on Phenylboronate–Agarose. The tryptic digest of the [^{3}H]N_3-NAD-treated BDH was applied to the phenylboronate–agarose column (5.6 × 84 mm) equilibrated with 50 mM potassium phosphate, pH 8.0. The column was first eluted with the same phosphate buffer. After the 280-nm absorbance of the eluate diminished to the background level and became constant, the elution medium was changed to distilled water. Fractions of 1 mL each were collected and sampled (10-μL aliquots) for radioactivity measurement. The radioactive fractions eluted with phosphate buffer and distilled water were pooled separately and lyophilized.

Peptide Purification by FPLC. For isolation of peptides, the Pharmacia FPLC system and the reverse-phase column Pep RPC HR 5/5 (5 × 50 mm) were used. Each of the two pooled and lyophilized fractions from the previous step was dissolved in 0.4 mL of 5 mM potassium phosphate, pH 6.5, and applied to a column equilibrated with the same buffer. Peptides were eluted by a linear gradient of acetonitrile. The fractions with the highest radioactivity were collected, lyophilized, dissolved in 0.4 mL of 10 mM HCOOH/NH_4OH, pH 4.0, and applied to a second reverse-phase column equilibrated in the ammonium formate buffer. Peptides were eluted as before. The peak fractions with the highest radioactivity were collected and lyophilized. The tryptic digest of carboxymethylated BDH not photolabeled with [^{3}H]N_3-NAD was also subjected to chromatography by FPLC, using the same conditions as described above, except that the phenylboronate column chromatography step was omitted.

Sequence and Amino Acid Analysis of Peptides. The amino acid sequence of the peptides was determined as described by others (Hewick et al., 1981; Hunkapiller & Hood, 1983). For amino acid analyses, the peptides in 6 N HCl were sealed in an ampule under vacuum and hydrolyzed at 110 °C for 24 h. Amino acid analysis was performed on a Beckman Model 121-M amino acid analyzer.

Results

Fractionation of the Tryptic Digest of BDH Labeled with [^{3}H]N_3-NAD. Figure 1 shows the elution pattern of the tryptic peptides of BDH photolabeled with [^{3}H]N_3-NAD from the phenylboronate–agarose column. This column binds structures having vicinal diols under alkaline, but not acid, conditions (Schott et al., 1973; Annamalai et al., 1979). Hence, the modified BDH peptide containing covalently bound NAD would be expected to bind to the column at pH 8.0. As seen in Figure 1, two protein peaks were eluted. The first peak that did not bind to the column and was washed through with 50 mM potassium phosphate, pH 8.0, contained most of the tryptic digest and about 15% of the recovered radioactivity. The second peak that was bound to the column and was eluted

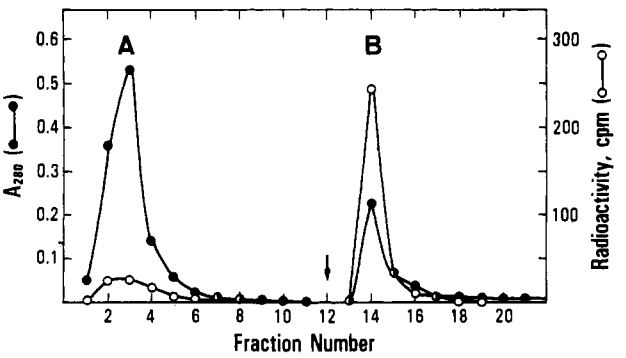

FIGURE 1: Affinity chromatography on phenylboronate–agarose of BDH tryptic peptides modified with [^{3}H]N_3-NAD. Peptides from the tryptic digest of BDH modified with [^{3}H]N_3-NAD (1.5 mg of protein) were applied in 1.0 mL of 50 mM ammonium bicarbonate to a phenylboronate–agarose 30 column (5.6 × 84 mm) equilibrated in 50 mM potassium phosphate, pH 8.0. The column was washed with the same buffer, then with distilled water at the point indicatd by an arrow. Fractions of 1.0 mL were collected and analyzed for absorbance at 280 nm (●), and for radioactivity (○), using 10-μL aliquots. Fractions 2–6 (peak A) and 14–18 (peak B) were separately pooled and lyophilized. Recovery of radioactivity was 15% in peak A and 48% in peak B.

with distilled water (pH ~5.5) contained 48% of the recovered radioactivity from [^{3}H]N_3-NAD. The reason that some radioactivity appeared in the first peak is that the ester linkage between NAD and arylazido-β-[^{3}H]alanine is susceptible to hydrolysis under the alkaline conditions used for carboxymethylation and trypsinolysis of the labeled enzyme (Jeng & Guillory, 1975; Chen & Guillory, 1977). Thus, a fraction of the labeled enzyme would be expected to lose NAD but retain radioactivity from the remainder of the probe. Peptides that had lost NAD but retained the remainder of the photolyzed [^{3}H]N_3-NAD would be expected to have radioactivity but not bind to phenylboronate–agarose. As will be seen, this was indeed the reason for the appearance of radioactivity in the flow-through fraction of Figure 1. For convenience, the flow-through and the water-eluted material of Figure 1 will be referred to as peaks A and B, respectively.

Panels A and B, respectively, of Figure 2 show the resolution pattern of peaks A and B of Figure 1 upon reverse-phase chromatography by FPLC, and panels A and B, respectively, of Figure 3 show the rechromatography of the peaks of panels A and B of Figure 2 with the highest radioactivity. As seen in Figure 2B, the single-step chromatography on phenylboronate–agarose resulted in extensive purification of the peptide containing bound [^{3}H]N_3-NAD. The peak fractions of panels A and B of Figure 3 that exhibited the highest radioactivity (peptides A and B) were collected for amino acid and sequence analyses. When unlabeled BDH was digested with trypsin and fractionated by FPLC, a peptide pattern similar to that shown in Figure 2A was obtained, except that the radioactive double peak was absent, and the peak marked by an arrow in Figure 2A was present at roughly twice the intensity. Thus, it was assumed that this peak may be the unlabeled tryptic peptide corresponding to that which was labeled with [^{3}H]N_3-NAD. This peak of Figure 2A was, therefore, collected and rechromatographed. Two major peaks were eluted (data not shown). The larger peak (peptide C) had the same amino acid sequence as peptides A and B.

Spectral Characteristics of Peptides A–C. The absorption spectra of comparable molar concentrations of peptides A–C are shown in Figure 4. Peptide B exhibited a peak at about 260 nm with a molar absorbance similar to that of N_3-NAD. This agreed with the chromatographic behavior of peptide B on phenylboronate–agarose, which suggested that peptide B

Table I: Amino Acid Composition of [^{3}H]N_3-NAD-Modified Peptide B and of Peptide C of BDH

amino acid	peptide B[a]	peptide C[a]	amino acid	peptide B[a]	peptide C[a]
Lys	0.92 (1)[b]	1.00 (1)	Gly	1.18 (1)	1.05 (1)
His	0 (0)	0 (0)	Ala	0.06 (0)	0 (0)
Arg	0 (0)	0 (0)	Val	0.98 (1)	0.88 (1)
Cm-Cys	0 (0)	0.88 (1)	Met	1.10 (1)	1.00 (1)
Asp	1.08 (1)	0.95 (1)	Ile	0.45 (1)	0.91 (1)
Thr	2.69 (3)	2.78 (3)	Leu	0 (0)	0 (0)
Ser	3.93 (4)	3.43 (4)	Tyr	0.92 (1)	1.01 (1)
Glu	1.04 (1)	1.07 (1)	Phe	0 (0)	0 (0)
Pro	0.99 (1)	0.97 (1)			

[a] Values are in units of residues/peptide. [b] Values in parentheses are the nearest integers.

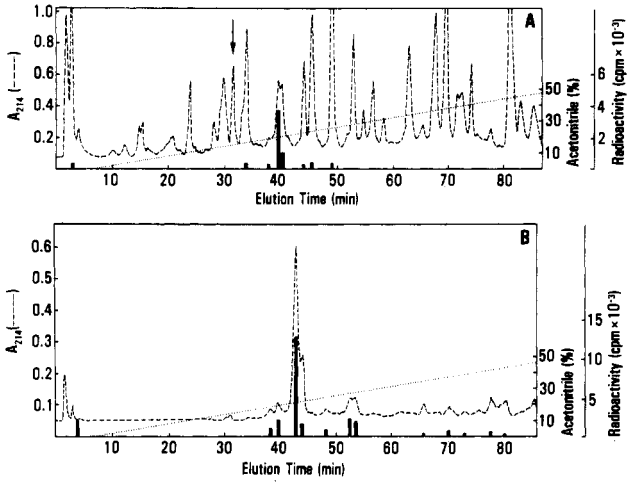

FIGURE 2: Separation of BDH tryptic peptides labeled with [^{3}H]N_3-NAD by FPLC reverse-phase chromatography. The reverse-phase column (Pep RPC HR 5/5) was equilibrated in 5 mM potassium phosphate, pH 6.5. Samples A and B from the phenylboronate–agarose column (Figure 1) were applied to separate reverse-phase columns (panels A and B, respectively), and the columns were eluted with a linear gradient of acetonitrile (dotted lines) as shown. Vertical bars represent the total radioactivity associated with the eluents collected for each peptide peak. The peak corresponding to that shown by an arrow in panel A was considerably greater in the elution pattern of the tryptic digest of unmodified apoenzyme (see text). Radioactivity recoveries in the major radioactive peaks of panels A and B were respectively 48% and 51%. Other details are described under Materials and Methods. NAD, N_3-NAD, and arylazido-β-alanine were eluted at 2, 33, and 21 min, respectively. The radioactivity eluted at 4 min in panel B is probably an altered form of the photoprobe that had dissociated from the labeled peptide.

contained bound NAD from photolyzed [^{3}H]N_3-NAD. Peptide A, which did not bind to phenylborate–agarose, exhibited a much smaller absorbance at about 260 nm, in agreement with the prediction that it had lost the NAD moiety of [^{3}H]N_3-NAD. Peptide C, which had no radioactivity and was assumed for the reasons stated above to be the unlabeled peptide corresponding to peptides A and B, showed no absorption peak at 260 nm and exhibited a small peak at about 275 nm in agreement with the presence in this peptide of tyrosine. The peak at 475 nm is that of free N_3-NAD (orange-red), and those at about 330 and 500 nm belong to the photolabeled peptides A and B (purple-red).

Amino Acid Composition and Sequence of Peptides A–C. Data for the amino acid composition and sequence of peptides B and C are given in Tables I and II, respectively. Data for peptide A (not shown) were essentially the same as those for peptide B. Results indicated the sequence Met-Glu-Ser-Tyr-Cys-Thr-Ser-Gly-Ser-Thr-Asp-Thr-Ser-Pro-Val-Ile-Lys. As seen in Table II, the cycle 5 residue could not be identified in the N_3-NAD-modified peptide B but was identified as carboxymethylcysteine in the unmodified peptide C. Similarly, one carboxymethylcysteine was identified in peptide C by

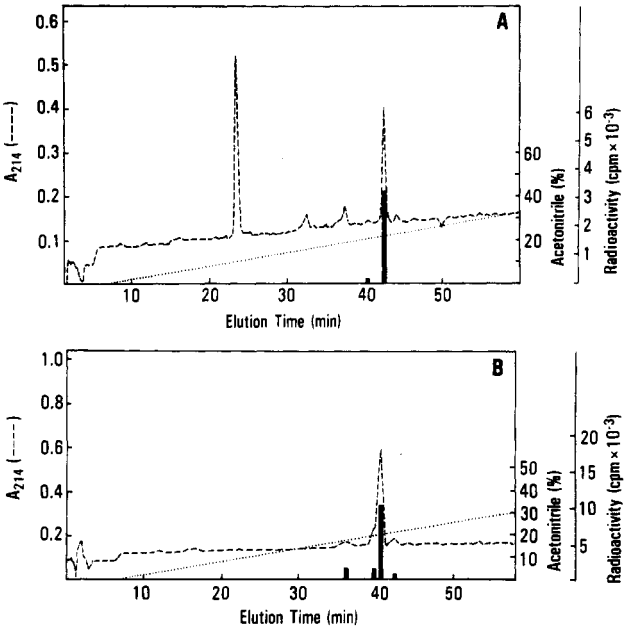

FIGURE 3: Purification of [^{3}H]N_3-NAD-labeled peptides of Figure 2 by reverse-phase FPLC chromatography. The same reverse-phase columns as in Figure 2 were used. The columns were equilibrated in 10 mM ammonium formate, pH 4.0. The major radioactive peaks from panels A and B of Figure 2 were separately applied to the reverse-phase columns, and the columns were eluted with a linear gradient of acetonitrile (dotted lines). Panels A and B show respectively the elution patterns of materials from panels A and B of Figure 2. Vertical bars show the total radioactivity associated with each peptide peak. Recoveries of radioactivity in the major radioactive peaks of panels A and B were respectively 82% and 75%. The final radioactivity recovery was 6% for peptide A and 18% for peptide B. Other details are described under Materials and Methods.

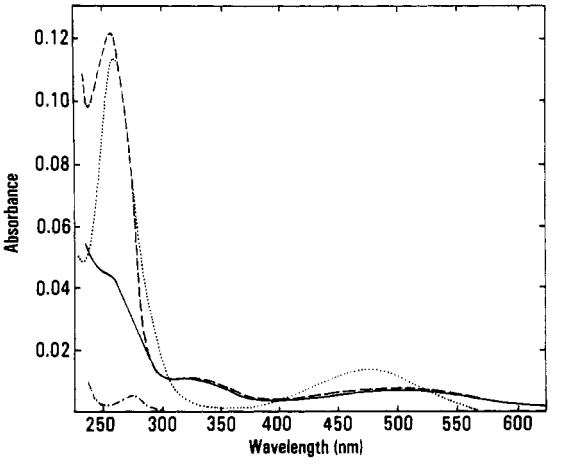

FIGURE 4: Absorption spectra of [^{3}H]N_3-NAD-modified peptides A and B and of peptide C. Peptides A (3.1 μM, —), B (3.4 μM, - - -), and C (3.8 μM, · · ·) and N_3-NAD (3.2 μM, ···) were dissolved in 50 mM potassium phosphate, pH 6.5, and their spectra were recorded by an Aminco DW-2a dual-wavelength spectrophotometer.

Table II: Amino Acid Sequence of [^{3}H]N_3-NAD-Modified Peptide B and of Peptide C of BDH[a]

	peptide B		peptide C	
cycle	amino acid	amount (pmol)	amino acid	amount (pmol)
1	Met	1350	Met	9830
2	Glu	680	Glu	7430
3	Ser	54	Ser	760
4	Tyr	430	Tyr	5420
5	*b*		Cm-Cys	4170
6	Thr	470	Thr	1800
7	Ser	47	Ser	400
8	Gly	430	Gly	4000
9	Ser	10	Ser	290
10	Thr	82	Thr	860
11	Asp	170	Asp	1020
12	Thr	35	Thr	650
13	Ser	*c*	Ser	87
14	Pro	51	Pro	530
15	Val	64	Val	770
16	Ile	59	Ile	560
17	*d*		Lys	290

[a] The amounts of peptides B and C analyzed were respectively 2 (3000 cpm) and 12 nmol. [b] In FPLC chromatography, no peak corresponding to an authentic phenylthiohydantoin derivative of any known amino acid was observed. [c] The amount was too small to be determined. [d] Could not be identified.

amino acid analysis, but not in peptide B (Table I). These results indicated, therefore, that cysteine in the above peptide was the residue modified by photoactivated [^{3}H]N_3-NAD.

Most of the radioactivity from the label was found in the Polybrene filter used in the sequencer, possibly because the adduct was too polar to be extracted by the solvent used (Hollemans et al., 1983; Knight & McEntee, 1985). However, there was a small amount of radioactivity (~2% of the total radioactivity applied) in the fifth cycle cleavage product, which agreed with the above conclusion regarding the site of attachment of photoactivated [^{3}H]N_3-NAD. As seen in Table II, recovery of serine during Edman degradation was low, which is a known problem in this and similar procedures (Edman & Begg, 1967; Chang, 1983). Data on the amino acid analysis of peptides A–C clearly indicated, however, the presence of four serines (recovery of 86–98%) in each peptide.

Discussion

The heptadecapeptide of BDH isolated after photoaffinity labeling with the substrate analogue [^{3}H]N_3-NAD and tryptic digestion of the protein was shown to have the sequence Met-Glu-Ser-Tyr-Cys-Thr-Ser-Gly-Ser-Thr-Asp-Thr-Ser-Pro-Val-Ile-Lys, and cysteine was the residue modified by the photoprobe. This short segment of the enzyme is marked by the following features: the presence of 8 hydroxy amino acids (4 serines, 3 threonines, and 1 tyrosine), symmetry of the hydroxy amino acids around glycine, and the preponderance of uncharged amino acid residues (14 of the 17 residues). The occurrence of 14 uncharged residues in this heptadecapeptide is consistent with the possibility that this peptide might constitute a portion of the adenosine-binding site of NAD (Rossman et al., 1975). The fact that the photoactivatable arm of N_3-NAD, namely, arylazido-β-alanine, is attached to a hydroxyl group of the ribose moiety of the adenosine portion of NAD (Chen & Guillory, 1977) agrees with this possibility. Furthermore, the remarkable occurrence of 8 hydroxy amino acids located around a glycine might be compared with data on the ATP-binding site of the mitochondrial adenosinetriphosphatase (ATPase). In the catalytic β subunit of bovine mitochondrial ATPase, photoactivated 8-azido-[2-^{3}H]ATP labels lysine-301, isoleucine-304, and tyrosine-311 (Hollemans et al., 1983). This region of the β subunit, i.e., from residue

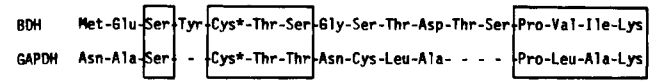

FIGURE 5: Comparison of the tryptic peptides of BDH and GAPDH labeled with [^{3}H]N_3-NAD. The cysteines marked with asterisks are the sites of [^{3}H]N_3-NAD insertion. In lobster GADPH, the second Leu (in the third box) is replaced by Val (Moras et al., 1975).

284 to residue 306, contains 2 glycines, 2 serines, and 7 threonines, all of which are conserved in the β subunits from several bacterial and plant sources (Falk et al., 1985).

While there was no sequence homology between the BDH peptide described above and LDH and MDH from several different sources (Birktoft et al., 1982), the following point may be of interest. It has been demonstrated that glyceraldehyde-3-phosphate dehydrogenase contains an essential cysteine (Cys-149) at the nicotinamide-binding region of the active site (Moras et al., 1975). Chen and co-workers have shown recently that Cys-149 of the rabbit muscle GAPDH is modified by photolabeling with tritiated arylazido-β-alanyl-NAD (Chen et al., unpublished results). A tryptic peptide containing the modified Cys-149 was isolated and sequenced by this group, and Figure 5 shows the degree of homology between the N_3-NAD-labeled peptides from BDH and GAPDH. These results might favor the possibility that the BDH peptide labeled with N_3-NAD represents a part of the nicotinamide-binding site of BDH.

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Registry No. BDH, 9028-38-0; N_3-NAD, 64700-08-9; NAD, 53-84-9; NADH, 58-68-4; L-Cys, 52-90-4.

References

Annamalai, A. E., Pal, P. K., & Colman, R. F. (1979) *Anal. Biochem. 99*, 85–91.

Baginski, E. S., Foa, P. P., & Zak, B. (1967) *Clin. Chem. (Winston-Salem, N.C.) 13*, 326–332.

Banaszak, L. J., & Bradshaw, R. A. (1975) *Enzymes (3rd Ed.) 11*, 369–396.

Berrez, J.-M., Latruffe, N., & Gaudemer, Y. (1984) *Biochem. Int. 8*, 697–706.

Birktoft, J. J., Fernley, R. T., Bradshaw, R. A., & Banaszak, L. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6166–6170.

Chang, J. Y. (1983) *Methods Enzymol. 91*, 455–466.

Chen, S., & Guillory, R. J. (1977) *J. Biol. Chem. 252*, 8990–9001.

Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.

Falk, G., Hampe, A., & Walker, J. E. (1985) *Biochem. J. 228*, 391–407.

Fleer, E., & Fleischer, S. (1983) *Biochim. Biophys. Acta 749*, 1–8.

Fleer, E. A. M., McIntyre, J. O., & Fleischer, S. (1984) *Biochemistry 23*, 5142–5147.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990–7997.

Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossman, M. G. (1975) *Enzymes (3rd Ed.) 11*, 191–292.

Hollemans, M., Runswick, M. J., Fearnley, I. M., & Walker, J. E. (1983) *J. Biol. Chem. 258*, 9307–9313.

Hunkapiller, M. W., & Hood, L. E. (1983) *Methods Enzymol. 91*, 486–493.

Jeng, S. J., & Guillory, R. J. (1975) *J. Supramol. Struct. 3*, 448–468.

Knight, K. L., & McEntee, K. (1985) *J. Biol. Chem. 260*, 10185–10191.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Maurer, A., McIntyre, J. O., Churchill, S., & Flesicher, S. (1985) *J. Biol. Chem. 260*, 1661-1669.
Moras, D., Olsen, K. W., Sabesan, M. N., Buehner, M., Ford, G. C., & Rossman, M. G. (1975) *J. Biol. Chem. 250*, 9137-9162.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346-356.
Phelps, D. C., & Hatefi, Y. (1981a) *Biochemistry 20*, 453-458.
Phelps, D. C., & Hatefi, Y. (1981b) *Biochemistry 20*, 459-463.
Prasad, P. V., & Hatefi, Y. (1986) *Biochemistry 25*, 2459-2464.
Rossman, M. G., Liljas, A., Branden, C.-I., & Banaszak, L. J. (1975) *Enzymes (3rd Ed.) 11*, 61-102.
Schott, H., Rudloff, E., Schmidt, P., Roychoudhury, R., & Kossel, H. (1973) *Biochemistry 12*, 932-938.
Yamaguchi, M., Chen, S., & Hatefi, Y. (1985) *Biochemistry 24*, 4912-4916.

Restrained Refinement of the Monoclinic Form of Yeast Phenylalanine Transfer RNA. Temperature Factors and Dynamics, Coordinated Waters, and Base-Pair Propeller Twist Angles†

E. Westhof
Institut de Biologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique, F-67084 Strasbourg Cedex, France
M. Sundaralingam*
Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706
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ABSTRACT: The structure of yeast phenylalanine transfer RNA in the monoclinic form has been further refined by using the restrained least-squares method of Hendrickson and Konnert. For the 4019 reflections between 10 and 3 Å, with magnitudes at least 3 times their standard deviations, the *R* factor is 16.8%. The variation of the atomic temperature factors along the sequence indicates that the major flexibility regions are the amino acid and anticodon stems. The two strands of the amino acid helix exhibit large differential temperature factors, suggesting partial uncoiling or melting of the helix. In this work, the occupancy of all atoms was also varied. Residues D16 and D17 of the dihydrouridine loop as well as U33 and G37 of the anticodon loop have occupancies around 70%, indicating some local disorder or large-scale mobility at these positions. One hundred fifteen solvent molecules, including five magnesium ions, were found in difference maps. The role of several water molecules is clearly related to the stabilization of the secondary and tertiary interactions. The gold sites, which were not previously discussed, are described and show an energetically favored binding mode similar to that of cobalt and nickel complexes with nucleotides.

Yeast phenylalanine transfer RNA (tRNA) crystallizes in two forms: monoclinic (Ichikawa & Sundaralingam, 1972; Ladner et al., 1972) and orthorhombic (Kim et al., 1971). Both are closely related forms with similar unit cell dimensions for the *a* and *b* axes (56.3 and 33.4 Å, respectively) while the *c* axis is about 2.5 times shorter in the monoclinic (63.0 Å) than in the orthorhombic (161.6 Å). The orthorhombic form has been refined with the program COREL S (Sussman et al., 1977) to 19.8% with individual atomic temperature factors or to 23.1% with group temperature factors for 8426 data up to 2.7-Å resolution (Sussman et al., 1978). The monoclinic form was refined by the MRC group using the Jack–Levitt refinement method (Jack & Levitt, 1978) to an *R* factor of 21% for 8006 data up to 2.5-Å resolution (Hingerty et al., 1978). The Madison group refined the monoclinic form using 6542 data up to 2.7 Å with difference Fourier methods down to 31.2% (Stout et al., 1978). Here, we report further refinement studies on the monoclinic form using the restrained refinement method of Hendrickson and Konnert (1980). This new refinement was performed in order to remove from the comparisons, between the structures of transfer RNA molecules known at high resolution, artifacts that might be introduced by different refinement methods. Extensive and precise comparisons between transfer RNA molecules are called for in view of the recent results obtained with yeast tRNA-Asp (Moras et al., 1985; Westhof et al., 1985). These results suggest that the structure of yeast $tRNA^{Asp}$ is a model for a tRNA molecule bound to a messenger codon triplet while that of yeast $tRNA^{Phe}$ is a model for the unbound tRNA molecule. This interesting observation has led to specific concern about the effects of crystallization conditions and of crystalline packing on temperature factors and dynamics. The results of the present refinement offer new insights on the yeast $tRNA^{Phe}$ molecule, and those will be principally discussed in this report.

Experimental Procedures

Data Collection and Refinement. The data used in this work were collected on a CAD4 diffractometer (Stout et al., 1978). The data were scanned out to 2.7 Å, and 8300 reflections were measured. Of these, 4019 reflections between 10 and 3 Å were above the 3σ level, and these were used in the present structure refinement.

The structure was refined by using the Konnert–Hendrickson restrained program (Hendrickson & Konnert, 1980;

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