Biochemistry

 Volume 32, Number 11 March 23, 1993

Accelerated Publications

Creation of an NADP-Dependent Pyruvate Dehydrogenase Multienzyme Complex by Protein Engineering†

J. Antonio Bocanegra, Nigel S. Scrutton, and Richard N. Perham*

Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

Received November 24, 1992

ABSTRACT: Systematic replacement of a set of amino acids in the $\beta\alpha\beta$-fold of the NAD-binding domain of *Escherichia coli* dihydrolipoamide dehydrogenase has been used to convert its coenzyme specificity from NAD to NADP. After comparison with the homologous enzyme glutathione reductase, Glu 203 was replaced with a valine residue, thereby eliminating the potential to form hydrogen bonds with the 2′- and 3′-OH groups of the adenine ribose in NAD. Similarly, Met 204, Pro 210, Phe 205, and Asp 206 were replaced by an arginine, an arginine, a lysine, and a histidine residue, respectively, to provide a nest of positive charge to accommodate the 2′-phosphate group of the incoming NADP. In addition, Gly 185 and Gly 189 in the $\beta\alpha\beta$ motif were replaced with alanine residues to facilitate the positioning of the newly introduced Val 203 by allowing a flip of the peptide bond between residues Gly 180 and Gly 181. Wild-type dihydrolipoamide dehydrogenase is inactive with NADP, but the mutant enzyme displayed high levels of activity with this coenzyme, the values of K_m, k_{cat}, and k_{cat}/K_m comparing favorably with those found for the wild-type enzyme operating with NAD. The mutant enzyme was also capable of assembly in vitro to form an active pyruvate dehydrogenase multienzyme complex, the coenzyme specificity of which reflected that of its dihydrolipoamide dehydrogenase component. These experiments should make it possible now to study the effects in vivo of requiring a crucial catabolic enzyme to function with the wrong coenzyme, an important extension of protein engineering into the living cell.

In biological systems, reductive endergonic reactions are generally catalyzed by enzymes that utilize NADPH as cofactor, whereas oxidative exergonic reactions are almost always catalyzed by enzymes that exhibit a marked preference for NAD (Stryer, 1988). The relevant enzymes must therefore discriminate between these structurally similar coenzyme molecules, which differ only by the presence of a phosphate group esterified to the 2′-hydroxyl group of the AMP moiety of NADP. It has proved possible to switch the coenzyme specificity of *Escherichia coli* glutathione reductase (EC 1.6.4.2) from NADP to NAD by systematic replacement of a set of amino acids in a $\beta\alpha\beta$ "fingerprint" motif in the NADP-binding domain of the enzyme (Scrutton et al., 1990). In principle, and because the NADP-binding domain of glutathione reductase (Karplus & Schulz, 1987; Ermler & Schulz, 1991) is a typical Rossmann fold (Rossmann et al., 1975; Wierenga et al., 1985), these mutations should constitute the basis of a general set of rules for manipulating the coenzyme specificity of NAD(P)-dependent oxidoreductases.

Dihydrolipoamide dehydrogenase (EC 1.8.1.4) is, like glutathione reductase, a member of the family of flavoprotein disulfide oxidoreductases (Williams, 1992). It is a component (E3) of the 2-oxo acid dehydrogenase multienzyme complexes [for review, see Patel and Roche (1990) and Perham (1991)],

† This work was supported by the Science and Engineering Research Council. J.A.B. is a Research Fellow supported by Programa Nacional de Becas de Formacion de Personal Investigador en el Extranjero, Ministerio de Educacion y Ciencia, España. N.S.S. is a Royal Society 1983 University Research Fellow and a Research Fellow of St. John's College, Cambridge.

* To whom correspondence should be addressed.

0006-2960/93/0432-2737$04.00/0

in which it catalyzes the NAD-dependent oxidation of enzyme-bound dihydrolipoamide (Reed, 1974). The amino acid sequences of *E. coli* glutathione reductase (Greer & Perham, 1986) and dihydrolipoamide dehydrogenase (Stephens et al., 1983) are known, as are the crystal structures of glutathione reductase from human red blood cells (Karplus & Schulz, 1987) and *E. coli* (Ermler & Schulz, 1991) and dihydrolipoamide dehydrogenase from *Azotobacter vinelandii* (Mattevi et al., 1991) and *Pseudomonas putida* (Mattevi et al., 1992).

We describe here the systematic conversion of the NAD-dependent enzyme, *E. coli* dihydrolipoamide dehydrogenase, into one that rejects NAD but is fully active with NADP. We have also examined the ability of the mutated enzyme to assemble into a pyruvate dehydrogenase multienzyme complex and the coenzyme specificity of the assembled product.

MATERIALS AND METHODS

Materials. Complex bacteriological media were from Difco Laboratories, and all media were prepared as directed in Maniatis et al. (1982). [^{35}S]dATP-αS triethylammonium salt (400 Ci/mmol) for DNA sequencing was supplied by Amersham International. Ethidium bromide, NAD, and NADP were from Sigma Chemical Co. Ultrapure agarose and CsCl were from Bethesda Research Laboratories. The restriction enzymes *Eco*RI and *Hin*dIII were purchased from New England Biolaboratories; calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. T4 DNA ligase and T4 polynucleotide kinase were from Amersham International. All other chemicals were of analytical grade. Glass-distilled water was used throughout. *E. coli* strain JRG1342 K12, Δ(*aro*P-*lpd*), DE74, *met*B1, *met*105, *azi*, *pox*, *pps*1, *tsx*87, *ton*, *rel*A1, *rps*L(Str^R), *rec*A1, was provided by Prof. J. R. Guest (Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, U.K.).

Site-Directed Mutagenesis and DNA Sequencing. Site-directed mutagenesis was carried out on a derivative of M13 containing the noncoding strand of the *lpd* gene (Stephens et al., 1983; N. S. Scrutton and R. N. Perham, unpublished work). Mutants were constructed by means of the phosphorothioate method (Taylor et al., 1985) as marketed by Amersham International, using the following mutagenic oligonucleotides: mutant A (E203V/M204R/F205K/D206H/P210R), 5′-ATTGACGTGGTTGTGCGGAAACACCAG-GTTATCCGTGCAGCTGACA-3′; mutant B (G185A/G189A), 5′-GGTATCATCGCCCTGGAAATGGCTACCG-TTTACC-3′. Using the latter oligonucleotide and single-stranded template encoding mutant A, a third mutant, C, was isolated that carries all seven mutations in the same gene. Putative mutants were screened directly by dideoxy sequencing with the T7 system marketed by Pharmacia.

Plasmid Construction. Plasmid or bacteriophage RF DNA was prepared by CsCl density centrifugation as described by Maniatis et al. (1982). Restriction endonuclease digestions were carried out as recommended by the enzyme suppliers. The mutated *lpd* genes were isolated by restricting bacteriophage RF DNA with *Eco*RI and *Hin*dIII, and the *lpd* gene fragment (6.2 kbp) was subcloned into the expression vector pKK223-3, as described elsewhere (Deonarain et al., 1989).

Purification of Enzymes. Wild-type and mutant dihydrolipoamide dehydrogenases were purified from the *lpd*-deletion strain JRG1342 of *E. coli* (Guest et al., 1985), transformed with the appropriate expression plasmid. The method of purification was similar to that used for the related flavoprotein oxidoreductase, glutathione reductase (Deonarain et al., 1989). The wild-type *E. coli* pyruvate dehydrogenase complex and the E1·E2 subcomplex from which the E3 component had been stripped were prepared as described by Perham et al. (1981). Pyruvate dehydrogenase complexes were reassembled by adding wild-type or mutant dihydrolipoamide dehydrogenase components. The wild-type complex and the E1·E2 subcomplex were prepared from *E. coli* as described previously (Perham et al., 1981). Complexes were reassembled by adding wild-type or mutant dihydrolipoamide dehydrogenase to the E1·E2 subcomplex in 100 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl, 2 mM EDTA, and 1 mM sodium azide. After incubation at 0–4 °C for 24 h, unbound dihydrolipoamide dehydrogenase was removed by gel filtration (Superose 6) in the same buffer, and the fractions containing the complex were pooled. Assembly was checked by submitting samples to electrophoresis in 12.5% SDS–polyacrylamide gels (Laemmli, 1970).

Measurement of Kinetic Parameters. Dihydrolipoamide dehydrogenase was assayed as described elsewhere (Sahlman & Williams, 1989) using an Applied Photophysics stopped-flow spectrophotometer. The apparent kinetic parameters were determined using 100 μM dihydrolipoamide and concentrations of NAD(P) ranging from 20 μM to 1 mM. All kinetic parameters were evaluated by nonlinear least-squares regression analysis to data points that are the mean of at least ten reactions. The values of k_{cat}/K_m for mutants A and C were determined as the slope of the first-order segment of the plot of velocity against NAD concentration. The specific catalytic activities of assembled pyruvate dehydrogenase complexes were determined as described previously (Danson & Perham, 1976). Protein was estimated by means of a micro method (Bradford, 1976) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Creation of a Binding Site for the 2′-Phosphate of NADP. In choosing the locations and types of mutation that might switch the coenzyme specificity of *E. coli* dihydrolipoamide dehydrogenase from NAD to NADP, we were guided by alignments of its amino acid sequence (Stephens et al., 1983) with the sequences of other members of the family of homologous flavoprotein disulfide oxidoreductases (Perham et al., 1991; Williams, 1992), notably the NADP-dependent *E. coli* glutathione reductase (Greer & Perham, 1986; Scrutton et al., 1990), as shown in Table I. Glutathione reductase (Karplus & Schulz, 1987; Ermler & Schulz, 1991) and dihydrolipoamide dehydrogenase (Mattevi et al., 1991) have very similar three-dimensional structures, as implied by the high degree of similarity in their amino acid sequences. Our previous studies on the coenzyme specificity of *E. coli* glutathione reductase (Scrutton et al., 1990) highlighted the importance of the side chains of a particular set of amino acids in the $\beta\alpha\beta$-fold of the NADP-binding domain (Rossmann et al., 1975; Wierenga et al., 1985): among them, two arginine residues (Arg 198 and Arg 204), a lysine residue (Lys 199), and a histidine residue (His 200) near the end of the second β-strand provide a nest of positive charge to accommodate the negative charge of the 2′-phosphate of NADP. Their importance has been borne out by the X-ray crystallographic analysis of the NAD-dependent mutant of *E. coli* glutathione reductase (Mittl et al., 1993). We therefore introduced these four residues into the corresponding positions (Met 204, Pro 210, Phe 205, and Asp 206, respectively) of *E. coli* dihydrolipoamide dehydrogenase.

We also exchanged a glutamate residue (Glu 203) in dihydrolipoamide dehydrogenase for the valine residue that

Table I: Amino Acid Sequences of *E. coli* Dihydrolipoamide Dehydrogenase and Glutathione Reductase around the $\beta\alpha\beta$ Dinucleotide-Binding Fold[a]

Dihydrolipoamide dehydrogenase	180	**G**	**G**	**G**	I	L	**G**	L	E	M	**G**	T	V	Y	H	A	L	G	S	Q	I	D	V	V	**E**	**M**	**F**	D	Q	V	I	**P**	A	A	D
Mutant A																									**V**	**R**	**K**	**H**				**R**			
Mutant B							**A**				**A**																								
Mutant C							**A**				**A**														**V**	**R**	**K**	**H**				**R**			
Glutathione reductase	174	**G**	A	**G**	Y	I	**A**	V	E	L	**A**	G	V	I	N	G	L	G	A	K	T	H	L	F	**V**	**R**	**K**	**H**	A	P	L	**R**	S	F	D

[a] The number indicates the position in the primary structure of the first residue shown for each sequence. Sequence references: glutathione reductase, *E. coli* (Greer & Perham, 1986); dihydrolipoamide dehydrogenase, *E. coli* (Stephens et al., 1983).

Table II: Apparent Kinetic Parameters of the Wild-Type and Mutant Forms of Dihydrolipoamide Dehydrogenase

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
NAD			
wild type	141.2	176.4	1.25×10^6
mutant A			4.32×10^4
mutant B	879.2	26.3	3×10^4
mutant C			6.32×10^3
NADP			
wild type	no detectable activity		
mutant A	9.76	56.8	5.82×10^6
mutant B	no detectable activity		
mutant C	60.1	95.2	1.58×10^6

occupies the equivalent position at the end of the second β-strand of the same $\beta\alpha\beta$-fold in glutathione reductase (Table I). The carboxyl group of this side chain is thought to make hydrogen bonds with the 2′- and 3′-hydroxyl groups of the adenine ribose moiety of NAD, a feature common to many NAD-linked dehydrogenases possessing the archetypal dinucleotide-binding fold (Rossmann et al., 1975; Wierenga et al., 1985). It forms another important part of the means by which such enzymes discriminate in favor of NAD over NADP (Scrutton et al., 1990; Feeney et al., 1990; Fan et al., 1991; Mittl et al., 1993). The *E. coli* dihydrolipoamide dehydrogenase thus altered to create a potential binding site for the 2′-phosphate of NADPH therefore contained five mutations (E203V, M204R, F205K, D206H, and P210R); it is referred to as mutant A (Table I).

E. coli dihydrolipoamide dehydrogenase is subject to severe product inhibition by NADH, and the kinetic parameters of the wild-type and mutant enzyme were therefore measured in a stopped-flow system. The enzyme also exhibits a small positive cooperative effect with respect to the nicotinamide coenzyme (Sahlman & Williams, 1989), but the effect is so small that the kinetics approximate to hyperbolic behavior. This enabled accurate estimates to be made of the Michaelis constants (Table II). Mutant A was found to have its K_m for NAD raised to a level where it could not reliably be measured; the fall in k_{cat}/K_m was approximately 30-fold. Correspondingly, mutant A displayed high levels of activity with NADP as coenzyme. The values of K_m, k_{cat}, and k_{cat}/K_m compare favorably with those found for the wild-type enzyme operating with NAD.

Mutation of the GXGXXG Motif. At the first β–α turn of the $\beta\alpha\beta$-fold in the dinucleotide-binding domain found in almost all NAD-linked oxidoreductases (Rossmann et al., 1975), there is a highly conserved Gly-X-Gly-X-X-Gly sequence motif. NAD binds to such enzymes with its diphosphate bridge at the β–α turn; the first two glycine residues are conserved to permit the turn and to allow the positive dipole charge at the N-terminus of the helix to interact with the negatively charged phosphate groups (Wierenga et al., 1985). The same sequence motif is commonly found in NADP-linked oxidoreductases, except that often (but not always) the third glycine residue is replaced by an alanine residue and there is another alanine residue a further four positions along the helix (Hanukoglu & Gutfinger, 1989). This can be seen quite clearly in the amino acid sequences of the flavoprotein disulfide oxidoreductases (Table I).

In *E. coli* glutathione reductase, the Gly-X-Gly-X-X-Ala motif has a part to play in conferring the specificity for NADPH, since replacement of the alanine residue with glycine (A179G) was a contributor to the switch of specificity from NADPH to NADH (Scrutton et al., 1990). This is due to the loss of the Cβ atom in the A179G mutation allowing a flip of the peptide bond between Gly 174 and Ala 175, which in turn facilitates the formation of hydrogen bonds between the carboxyl group of another amino acid introduced for the purpose, Glu 197, with the 2′- and 3′-hydroxyl groups of the adenine ribose of NAD (Mittl et al., 1993). A similar conclusion has been reached from a general study of nicotinamide coenzyme-binding sites in oxidoreductases (Baker et al., 1992). For these reasons, therefore, we introduced the reverse mutation (G185A) into *E. coli* dihydrolipoamide dehydrogenase (Table I). We simultaneously introduced the other alanine residue (G189A in *E. coli* dihydrolipoamide dehydrogenase) found in the motif for some NADP-dependent enzymes (Hanukoglu & Gutfinger, 1989). This is the sequence in wild-type *E. coli* glutathione reductase (Table I) although the second alanine residue appears to play no part in determining coenzyme specificity (Scrutton et al., 1990). The mutated dihydrolipoamide dehydrogenase carrying these two altered residues (G185A, G189A) is referred to as mutant B.

For mutant B operating with NAD, there was a 40-fold fall in the value of k_{cat}/K_m, consisting of a 6-fold increase in K_m and a 7-fold decrease in k_{cat} (Table II). The mutant was inactive with NADP as coenzyme.

Combination of Mutations. On combining the two mutations in mutant B with the five mutations in mutant A, a further mutant (mutant C) was generated. For mutant C operating with NAD, the value of k_{cat}/K_m was found to have decreased a further 7-fold when compared with mutant A (Table II); with NADP, the value of K_m was found to have risen by about 6-fold and that of k_{cat} by 2-fold, but overall, as measured by the ratio of the values of k_{cat}/K_m for NAD and NADP, mutant C is approximately twice as selective for NADP over NAD as mutant A. Wild-type *E. coli* dihydrolipoamide dehydrogenase is inactive with NADP as coenzyme. The effect of these mutations, therefore, has been to switch the coenzyme specificity of dihydrolipoamide dehydrogenase from NAD to NADP and to produce a mutant enzyme that remarkably has kinetic parameters for NADP similar to those that the wild-type enzyme displays with NAD.

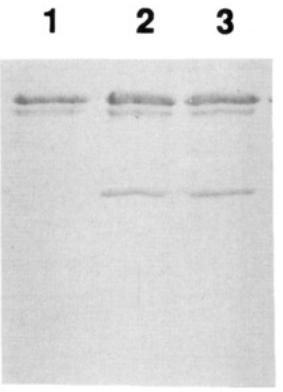

FIGURE 1: Reassembly of pyruvate dehydrogenase multienzyme complexes with wild-type and mutant dihydrolipoamide dehydrogenase components. Complexes were reassembled by adding wild-type or mutant dihydrolipoamide dehydrogenase (mutant C) to the wild-type E1·E2 subcomplex. Unbound dihydrolipoamide dehydrogenase was removed by gel filtration, and the complexes were submitted to SDS–polyacrylamide gel electrophoresis. Lanes: 1, E1·E2 subcomplex; 2, wild-type complex; 3, complex assembled with mutant C. Proteins were visualized by staining with Coomassie Brilliant Blue R250.

Table III: Catalytic Activities of the Assembled Wild-Type and Mutant Pyruvate Dehydrogenase Multienzyme Complexes

	specific catalytic activity of complex (units/mg)	
enzyme	NAD	NADP
wild type	20.5	no detectable activity
mutant C	0.53	19.4

Assembly into a Pyruvate Dehydrogenase Complex. In the cell, dihydrolipoamide dehydrogenase is a component of the 2-oxo acid dehydrogenase multienzyme complexes (Patel & Roche, 1990; Perham, 1991). It was of interest therefore to determine whether the mutant forms of dihydrolipoamide dehydrogenase could assemble into a multienzyme complex and confer on it specificity for NADP. Wild-type *E. coli* dihydrolipoamide dehydrogenase and mutant C were mixed in vitro with the E1·E2 subcomplex of the *E. coli* pyruvate dehydrogenase complex, and the assembled products were isolated by gel filtration (Figure 1). Analysis of the products by means of SDS–polyacrylamide gel electrophoresis revealed that in each instance an active complex was formed and that its coenzyme specificity reflected that of its dihydrolipoamide dehydrogenase component (Table III).

Conclusions. Systematic replacement of a set of amino acid residues in the $\beta\alpha\beta$-fold of the dinucleotide-binding domain of NADPH-dependent glutathione reductase converted it into an enzyme with a marked preference for NADH (Scrutton et al., 1990). By means of a comparable set of mutations, we have now been able to switch the coenzyme specificity of *E. coli* dihydrolipoamide dehydrogenase from NAD to NADP, demonstrating the generality of the approach. Moreover, the modified dihydrolipoamide dehydrogenase was able to assemble to form a fully active pyruvate dehydrogenase multienzyme complex. This enzyme complex catalyzes a crucial catabolic reaction, the oxidative decarboxylation of pyruvate to yield acetyl-CoA. These experiments should thus make it possible now to study the effects in vivo of requiring such an enzyme to function with the wrong coenzyme, an important extension of protein engineering into the living cell.

ACKNOWLEDGMENT

We thank Professor J. R. Guest, University of Sheffield, for supplying *E. coli* strain JRG 1342.

REFERENCES

Baker, P. J., Bitten, K. L., Rice, D. W., Rob, A., & Stillman, T. J. (1992) *J. Mol. Biol. 228*, 662–671.
Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.
Danson, M. J., & Perham, R. N. (1976) *Biochem. J. 159*, 677–682.
Deonarain, M. P., Berry, A., Scrutton, N. S., & Perham, R. N. (1989) *Biochemistry 28*, 9602–9607.
Ermler, J., & Schulz, G. E. (1991) *Proteins: Struct., Funct., Genet. 9*, 174–179.
Fan, F., Lorenzen, J. A., & Plapp, B. V. (1991) *Biochemistry 30*, 6397–6401.
Feeney, R., Clarke, A. R., & Holbrook, J. J. (1990) *Biochem. Biophys. Res. Commun. 188*, 667–672.
Greer, S., & Perham, R. N. (1986) *Biochemistry 25*, 2736–2742.
Guest, J. R., Lewis, H. M., Graham, L. D., Packman, L. C., & Perham, R. N. (1985) *J. Mol. Biol. 185*, 743–754.
Hanukoglu, I., & Gutfinger, T. (1989) *Eur. J. Biochem. 180*, 479–484.
Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 145*, 701–729.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mattevi, A., Schierbeek, A. J., & Hol, W. G. J. (1991) *J. Mol. Biol. 220*, 975–994.
Mattevi, A., Obmolova, G., Kalk, K. H., Sokatch, J., Betzel, C. H., & Hol, W. G. J. (1992) *Proteins: Struct., Funct., Genet. 13*, 336–351.
Mittl, P., Berry, A., Scrutton, N. S., Perham, R. N., & Schulz, G. E. (1993) *J. Mol. Biol.* (in press).
Patel, M. S., & Roche, T. E. (1990) *FASEB J. 4*, 3224–3233.
Perham, R. N. (1991) *Biochemistry 30*, 8501–8512.
Perham, R. N., Duckworth, H. W., & Roberts, G. C. K. (1981) *Nature 292*, 474–477.
Perham, R. N., Scrutton, N. S., & Berry, A. (1991) *BioEssays 13*, 515–525.
Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40–46.
Rossmann, M. G., Liljas, A., Bränden, C. I., & Banaszak, L. J. (1975) *Enzymes 11*, 61–102.
Sahlman, L., & Williams, C. H., Jr. (1989) *J. Biol. Chem. 264*, 8039–8045.
Scrutton, N. S., Berry, A., & Perham, R. N. (1990) *Nature 343*, 38–43.
Stephens, P. E., Lewis, H. M., Darlison, M. G., & Guest, J. R. (1983) *Eur. J. Biochem. 135*, 519–527.
Stryer, L. (1988) *Biochemistry*, W. H. Freeman & Co., New York.
Wierenga, R. K., de Maeyer, M. C. H., & Hol, W. G. J. (1985) *Biochemistry 24*, 1345–1357.
Williams, C. H., Jr. (1992) in *Chemistry and Biochemistry of Flavoproteins* (Müller, F., Ed.) Vol. III, pp 121–211, CRC Press, Boca Raton, FL.