Eur. J. Biochem. **267**, 929–934 (2000) © FEBS 2000

A change in reaction specificity of sheep liver serine hydroxymethyltransferase

Induction of NADH oxidation upon mutation of His230 to Tyr

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Both serine hydroxymethyltransferase and aspartate aminotransferase belong to the α-class of pyridoxal-5′-phosphate (pyridoxal*P*)-dependent enzymes but exhibit different reaction and substrate specificities. A comparison of the X-ray structure of these two enzymes reveals that their active sites are nearly superimposable. In an attempt to change the reaction specificity of serine hydroxymethyltransferase to a transaminase, His 230 was mutated to Tyr which is the equivalent residue in aspartate aminotransferase. Surprisingly, the H230Y mutant was found to catalyze oxidation of NADH in an enzyme concentration dependent manner instead of utilizing L-aspartate as a substrate. The NADH oxidation could be linked to oxygen consumption or reduction of nitrobluetetrazolium. The reaction was inhibited by radical scavengers like superoxide dismutase and D-mannitol. The K_m and k_{cat} values for the reaction of the enzyme with NADH were 74 μM and 5.2×10^{-3} s^{-1}, respectively. This oxidation was not observed with either the wild type serine hydroxymethyltransferase or H230A, H230F or H230N mutants. Thus, mutation of H230 of sheep liver serine hydroxymethyltransferase to Tyr leads to induction of an NADH oxidation activity implying that tyrosyl radicals may be mediating the reaction.

Keywords: serine hydroxymethyltransferase (SHMT); pyridoxal*P*; H230; NADH oxidation.

Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1), a pyridoxal 5′-phosphate (pyridoxal*P*)-dependent enzyme, catalyzes the conversion of serine and H_4-folate to glycine and 5,10-CH_2-H_4-folate [1]. Like other pyridoxal*P*-dependent enzymes, SHMT exhibits broad substrate and reaction specificity as it catalyzes aldolytic cleavage, decarboxylation, racemization and transamination reactions. Site-specific mutations of active site residues has led to a change in the reaction specificity of enzymes [2–6] including pyridoxal*P* containing enzymes such as aspartate aminotransferase (AATase), tyrosine phenol lyase (TPL), etc. Graber *et al*. [2] showed that the reaction specificity of AATase could be altered to that of a β-decarboxylase, by converting the Y225 to R and coupling it to another mutant, R386A. Mutation of the active site R386 to K altered the reaction and substrate specificity of AATase [5]. The mutant enzymes catalyzed the βdecarboxylation of L-aspartate and the racemization of amino acids at rates faster than that of the wild type enzyme. In the case of TPL, which normally catalyzes the β-elimination reaction of tyrosine, grafting of two amino acids of AATase thought to be important for recognition of dicarboxylic substrates enabled the mutant TPL to accept dicarboxylic amino acids as substrates without significant change in its reaction specificity [7]. H230 and R401 in sheep liver SHMT (scSHMT) correspond to Y225 and R386, respectively, of AATase. Introduction of a Y residue at position 230 in the active site of SHMT would probably mimic the active site geometry of AATase, as X-ray diffraction studies had indicated remarkable similarity in the active site configuration of the two enzymes [8]. This paper describes the induction of a new catalytic activity in scSHMT viz. NADH oxidation in the H230Y mutant of scSHMT.

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Abbreviations: pyridoxal*P*, pyridoxal-5′-phosphate; SHMT, serine hydroxymethyltransferase; hcSHMT, human liver cytosolic SHMT; H_4-folate, 5,6,7,8-tetrahydrofolate; scSHMT, sheep liver recombinant SHMT.

Enzyme: serine hydroxymethyltransferase (SHMT, EC 2.1.2.1)

Note: part of this work was presented at the 10th International Symposium on Vitamin B_6 and Carbonyl Catalysis and 4th meeting on PQQ and quinoproteins at Santa Fe, NM, USA, 31 October to 5 November 1999.
(Received 6 December 1999, accepted 13 December 1999)

EXPERIMENTAL PROCEDURES

Glycine, L-serine, NADH, 2-mercaptoethanol, folic acid, pyridoxal*P*, EDTA, L-aspartate, α-ketoglutarate and L-*allo*-threonine were obtained from Sigma Chemicals Co. St. Louis, MO, USA. [α-^{32}P]dATP (3000 Ci·mmol^{-1}) and L-[3-^{14}C] serine (55 mCi·mmol^{-1}), restriction endonucleases, Sequenase Version 2 DNA sequencing kit and DNA modifying enzymes were obtained from Amersham Pharmacia Biotech, Bucks, UK. [2–^{3}H]Glycine (41.1 Ci mmol^{-1}) was purchased from NEN Life Science Products, Inc. Boston, MA, USA. *Pfu* polymerase was purchased from Stratagene and Deep Vent polymerase from New England Biolabs, Inc., Beverly, MA, USA. Sephacryl S-200, Superose 12 HR 10/30 were purchased from Pharmacia, Uppsala, Sweden. The oligonucleotide primers were custom synthesized by Bangalore Genei Pvt. Ltd, India. H_4-Folate was prepared by the method of Hatefi *et al*. [9]. All other biochemicals used in this study were of the highest purity available.

Bacterial Strains and growth conditions

Escherichia coli DH5α was the recipient strain for the plasmids used in subcloning and sequencing. Strain BL21(DE3) pLysS

[10] was used for bacterial expression of pET constructs. Luria–Bertani medium or terrific broth with 50 $\mu g \cdot mL^{-1}$ of ampicillin was used for growing *E. coli* cells.

DNA manipulations

Plasmids were prepared by the alkaline lysis method as described by Sambrook *et al*. [11]. Restriction analysis and DNA ligations were carried out according to the manufacturer's instructions. Competent cells were prepared by the procedure of Alexander [12].

Site-directed mutagenesis

The H230Y mutant was constructed by the PCR based megaprimer method [13,14]. The oligonucleotides used for the generation of mutants were: H230Y–5′-ACATGGCATA-TATCAGCG-3′; M13 reverse primer (19-mer) – 5′ GGAAAC-AGCTATGACCATG-3′ and SHP1 (19-mer) – 5′-TATGG-CAGCTCCAGTCAAC-3′ corresponding to the 5′-end of the SHMT gene. The full length PCR product was subcloned into pUC19 at *Kpn*I and *Bam*HI sites. The clone was double digested with *Kpn*I and *Pml*I to obtain a 0.5-kb fragment which was swapped with the corresponding 0.5 kb fragment of the wild type clone, i.e. pETSH (SHMT gene in pET 3c). The entire 0.5 kb region was sequenced using Sequenase Version 2 DNA sequencing kit to confirm the presence of the desired mutation and the absence of nonspecific mutation(s).

Expression and purification of H230 mutant proteins

scSHMT and the mutant enzyme was purified by the protocol described earlier [15]. Briefly, the BL21(DE3) pLysS extracts were subjected to ammonium sulphate fractionation, CM-Sephadex and Sephacryl S-200 column chromatography. The fractions were pooled and precipitated with 65% ammonium sulphate and the pellet was resuspended in Buffer A (50 mM phosphate buffer, pH 7.4, 1 mM EDTA and 1 mM 2-mercaptoethanol). It was dialyzed against the same buffer with two changes and was used in all further studies. The amount of protein was estimated by the method of Lowry *et al*. [16].

Spectral measurements

The absorbance spectra of the wild type and mutant enzymes (5 μM) were recorded in a Shimadzu UV 160 A spectrophotometer, both in the presence and absence of ligands and at different time intervals, when necessary. The oligomeric status of the proteins was determined using a calibrated Superose 12 HR 10/30 column attached to a Pharmacia FPLC system.

Enzyme assays

Aldol cleavage in the presence of H_4-folate was monitored using L-[3–^{14}C]serine by the method of Manohar *et al*. [17]. Aldolytic cleavage of L-*allo*threonine was monitored as described by Jagath *et al*. [18].

Transamination of L-aspartate. Transamination of L-aspartate in presence of a-KG generates glutamate and oxaloacetate (OAA) as products. The reaction was followed by measuring the oxidation of NADH in the presence of malate dehydrogenase (MDH). The reaction was carried out at 37 °C for 15 min using 5 μM of protein along with 10 mM L-aspartate, 1 mM of α-KG, 250 μM of NADH and six units of MDH. The progress of the reaction was followed by the change in absorbance at 340 nm as a result of the oxidation of NADH to NAD.

Oxidation of NADH:

(*a*) *Absorbance spectroscopy.* The oxidation of NADH was followed by measuring the decrease in absorbance at 340 nm over a period of 15 min. The protein (5 μM) was incubated in buffer A with varying concentrations of NADH (0–500 μM) and the change in A_{340} per min was recorded. A double reciprocal plot of ΔA_{340} per min vs. the concentration of NADH was used to determine the K_m value for NADH. A molar extinction coefficient of 6220 $M^{-1} \cdot cm^{-1}$ was used to calculate the k_{cat} value for the reaction.

(*b*) *Oxygen consumption.* All measurements of oxygen consumption were carried out at 30 °C in a Gilson 5/6 H oxygraph fitted with a Clark electrode in a reaction vessel of 1.75 mL. Under the experimental conditions, the amount of oxygen consumed was standardized by the decrease in the amount of dissolved oxygen. A value of 0.224 mM was used for the concentration of dissolved oxygen at 30 °C. The total change in oxygen concentration was calculated and expressed in μM. A change in the dissolved oxygen concentration of 100 μM corresponds to 220 nmol in the total reaction mixture. The oxygen consumption during NADH oxidation reaction was monitored using 5 μM enzyme in buffer A in presence of 250 μM NADH.

(*c*) *NADH dependent dye reduction.* Artificial electron acceptors were used to monitor the NADH oxidation reaction by SHMT and its mutants. The proteins (5 μM) were incubated with 100 μM nitrobluetetrazolium (NBT) and 250 μM of NADH, at 37 °C and the change in absorbance at 560 nm was followed as a function of time. The reaction was also carried out following a preincubation with superoxide dismutase (5 μg) or D-mannitol (50 mM).

RESULTS

The wild type recombinant SHMT (scSHMT) and the mutant enzyme (H230Y) were tested for their physiological as well as newly generated catalytic activities, if any. Mutation of H230 to Y showed considerable loss ($\approx$ 90%) of H_4-folate dependent aldolytic cleavage of serine. The specific activity of H230Y SHMT was 0.52 $U \cdot mg^{-1}$ compared to a value of 4.8 $U \cdot mg^{-1}$ observed for scSHMT. The absorption spectrum of H230Y was identical to that for scSHMT (Fig. 1). But, the amount of quinonoid intermediate formed was very small compared to that observed in scSHMT and corresponded to the observed physiological activity of H230Y SHMT (Fig. 1). However, the mutation of H230 to Y affected the alternate reactions to varying extents.

Transamination of L-aspartate

AATase catalyzes the transamination of aspartate to glutamate using α-KG and yields the keto acid, OAA, as the product. As Y is present in an equivalent position in AATase, it was of interest to examine the transaminase activity of H230Y. However, the mutant enzyme failed to show an aspartate dependent decrease in absorbance at 340 nm (data not shown) suggesting that the transamination reaction was not occurring. Attempts to detect the presence of any glutamate generated in the mixture, by amino-acid analysis, were unsuccessful (data not shown).

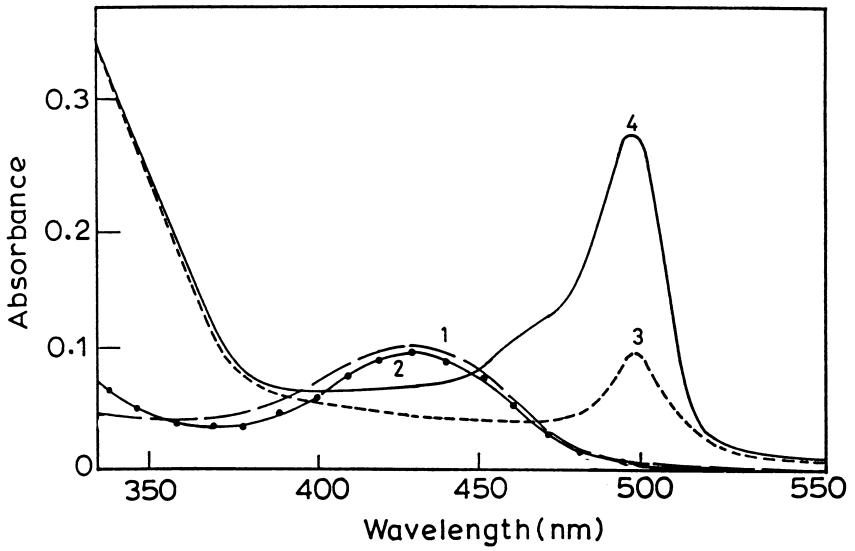

Fig. 1. The visible absorption spectra of scSHMT and H230Y mutant. Visible absorption spectra of scSHMT and H230Y (line 1) were recorded in a Shimadzu UV-160A spectrophotometer using 5 μM protein in Buffer A. The spectra were re-recorded upon the addition of 100 mM glycine (line 2) to scSHMT and H230Y followed by the addition of 0.18 mM H_4-folate (line 3: H230Y; line 4: scSHMT).

Interestingly, during our attempts to monitor changes in reaction specificity using L-*allo*-threonine or L-threonine, it was observed that NADH was oxidized even in the absence of added amino acid or coupling enzyme to H230Y mutant protein suggesting a change in reaction specificity.

Oxidation of NADH

When the H230Y mutant protein (5 μM) was incubated with 250 μM NADH in buffer A, at 37 °C a significant decrease in absorbance at 340 nm was observed (Fig. 2). The A_{340} value decreased from 0.85 to 0.236 units in 15 min. In a control experiment using scSHMT and other mutants such as H230A, H230N or H230F, the oxidation of NADH was not observed (Fig. 2). A boiled enzyme control also did not show any reaction. The formation of NAD^+ in the reaction was confirmed by reducing it with ethanol (50 μM) and ADH (100 $\mu g \cdot mL^{-1}$) which resulted in a sharp increase in A_{340} value (Fig. 2). This change in absorbance (from 0.26 to 0.52 units) corresponded to the amount of ethanol added to the reaction mixture. When H230Y was incubated with 250 μM of NADPH in buffer A at 37 °C, only a very small change in absorbance (ΔA_{340} = 0.03 in 15 min) was observed (Fig. 2) thus implying that the enzyme preferred NADH over NADPH as the substrate for oxidation. These observations suggested that mutation of H230 to Y had conferred on SHMT, an ability to oxidize NADH. The dependence of the reaction on the concentration of the enzyme was measured by varying the enzyme concentration in the range of 0–7.5 μM. The activity increased linearly up to 5 μM and plateaued thereafter. The kinetic constants for the interaction of H230Y with NADH were measured by varying the NADH concentration from 0 to 500 μM and measuring the initial velocity. The kinetic constants for H230Y were: K_m = 74 μM and $k_{cat} = 5.2 \times 10^{-3} \cdot s^{-1}$. The values were calculated by least square fit of the data. As there was hardly any reaction with scSHMT or other mutants (H230A, H230N or H230F), the kinetic constants could not be evaluated for these proteins. These results clearly demonstrate that H230Y SHMT is capable of NADH oxidation even in the absence of added electron acceptors thus implicating oxygen to be the electron acceptor. The NADH dependent oxygen consumption by scSHMT was very small ($< 1.0\ \mu M \cdot min^{-1}$), whereas in the presence of H230Y, it was 5.2 $\mu M \cdot min^{-1}$ (Fig. 3).

Reduction of artificial electron acceptors

The oxidation of NADH is, very often, linked to electron acceptors such as nitrobluetetrazolium (NBT). The unavailability

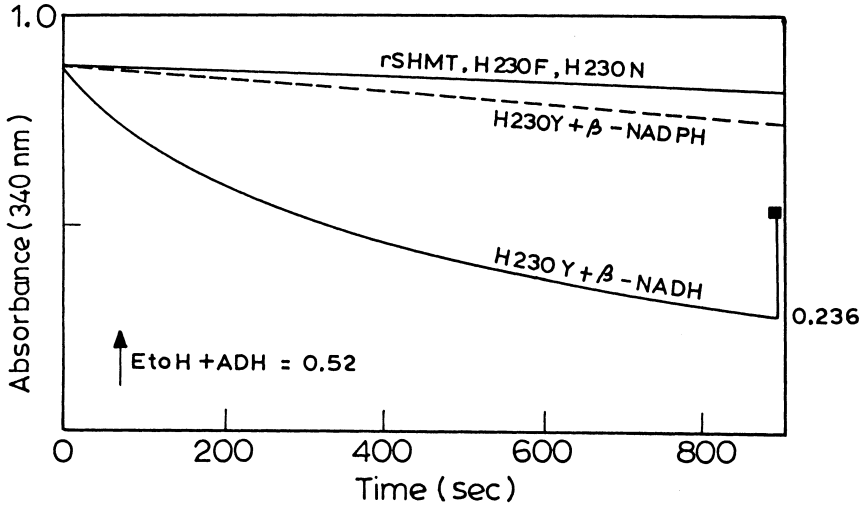

Fig. 2. Time course of NADH oxidation by scSHMT, H230Y and other mutants. The enzymes (5 μM), scSHMT, H230F, H230N and H230Y were incubated with 250 μM NADH in buffer A, at 37 °C and the change in absorbance at 340 nm was monitored (—). Line (– –) shows the reaction of H230Y with 250 μM of β-NADPH. The filled square (■) indicates the regeneration of NADH from NAD^+ upon addition of alcohol dehydrogenase (100 $\mu g \cdot mL^{-1}$) and ethanol (50 μM).

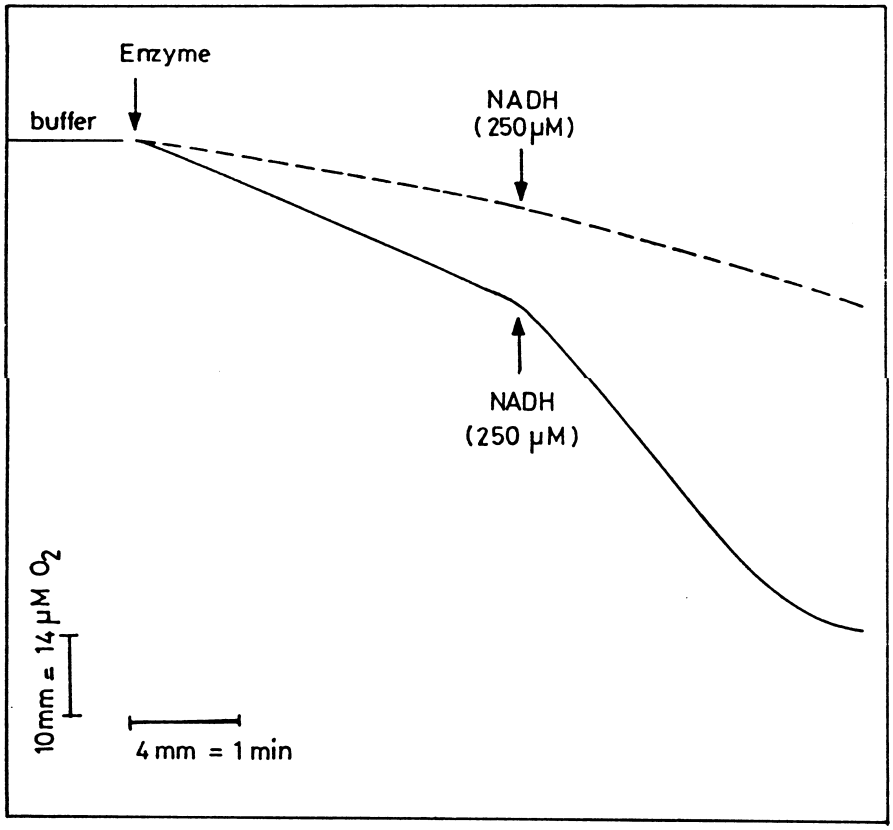

Fig. 3. H230Y dependent oxidation of NADH. The standard conditions of assay of oxidation of NADH coupled to oxygen uptake were followed. NADH was added at a concentration of 250 μM. The broken line represents the oxygen uptake observed in the presence of 5 μM scSHMT and the solid line shows the oxygen uptake in presence of 5 μM H230Y.

of facilities for anaerobic experiments precluded measuring the reaction under anaerobic conditions. Incubating the mutant enzyme, H230Y (5 μM), with 100 μM NBT followed by the addition of 250 μM NADH, resulted in the reduction of the dye (Fig. 4). An equal concentration of scSHMT or the other mutants of H230 failed to catalyze the NADH dependent dye reduction.

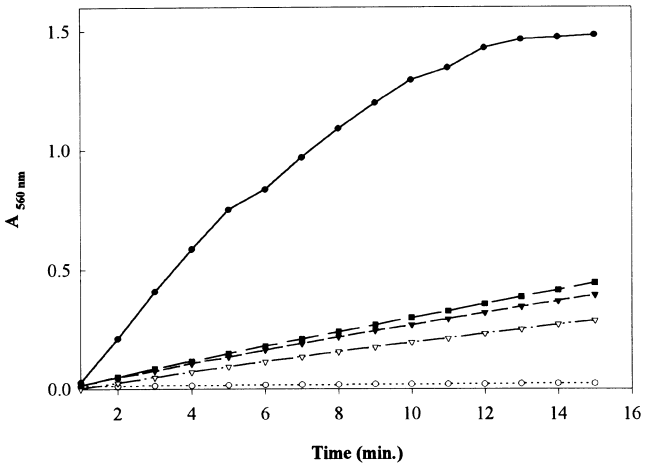

Fig. 4. The reduction of NBT by scSHMT and H230Y in the presence of NADH. 100 μM NBT along with 5 μM of H230Y was taken in buffer A and the reaction initiated by the addition of 250 μM NADH. The reaction was monitored by measuring the change in absorbance at 560 nm. Open circles (○) represent the reduction of NBT by scSHMT and other mutants (H230A, H230F and H230N) and closed circles (●) represent the reduction of NBT by H230Y. The inhibition of NADH oxidation by SOD (5 $\mu g \cdot mL^{-1}$) (■) or D-mannitol (50 or 200 mM, ▼ or ▽) was monitored by preincubating the protein with either SOD or D-mannitol for 5 min. and then following the reaction upon addition of NBT and NADH.

Effect of free radical scavengers

The enzymatic and nonenzymatic oxidation of NADH coupled to dye reduction proceeds, very often, through the intermediate formation of superoxide anions. The inhibition of the reaction by superoxide dismutase (SOD) has often been used as a diagnostic tool in many studies [19,20]. The protein (H230Y) was preincubated with 5 $\mu g \cdot mL^{-1}$ of SOD for 5 min and the reaction was initiated by the addition of 100 μM NBT and 250 μM NADH. It is evident from Fig. 4 that the rate of the reaction decreased substantially. The rate of the reaction ($\Delta A_{560\ nm} \cdot min^{-1}$), in the absence of SOD, was $0.17 \cdot min^{-1}$, whereas in the presence of 5 $\mu g \cdot mL^{-1}$ of SOD or 50 mM D-mannitol, a free radical scavenger, it was $0.025 \cdot min^{-1}$ and $0.02 \cdot min^{-1}$, respectively.

DISCUSSION

The selective binding of substrate(s) to an enzyme is a fundamental feature of enzyme-catalyzed reactions. A study of the molecular basis for substrate recognition has become more accessible following the advent of the methods of site-directed mutagenesis. The results presented in this paper demonstrate that mutation of H230 to Y in scSHMT leads to the generation of a new catalytic activity.

The transfer of the hydride ion from pyridine nucleotide involves a variety of acceptors ranging from disulphides [21], Fe-S clusters [22,23], flavins [24], cytochromes [25], heme prosthetic groups [26], etc. The transfer of the reducing equivalents to oxygen occurs in a step-wise manner leading

either to the formation of H_2O_2 or H_2O and in many instances, superoxide, hydroxyl radicals, etc. are key intermediates in this process. In oxygenases, where the atoms of oxygen are incorporated into the substrate, pyridine nucleotides provide the reducing equivalents essential for the reaction [27]. Another important class of reactions involving pyridine nucleotides is the reduction of a number of intermediates in the metabolic pathways of amino acids, carbohydrates and lipids such as pyruvate, oxalate, malate, etc. A survey of literature has failed to reveal the involvement of the oxidation/reduction of NADH in pyridoxal*P*-dependent enzymes, although NADH oxidation has been used to monitor half reactions of transamination, etc. by coupling the keto acid products to other enzyme systems.

The oxidation of NADH observed with H230Y mutant protein (Fig. 2) is very intriguing. The enzyme catalyzed oxidation reaction occurred at reasonable rates (k_{cat} = $5.2 \times 10^{-3} \cdot s^{-1}$). The reaction was found to occur under aerobic conditions as oxygen was shown to be an electron acceptor. The rates of oxygen consumption of H230Y were increased significantly in presence of NADH (Fig. 3). NBT was also found to be an effective acceptor (Fig. 4). The reaction was inhibited by SOD and D-mannitol (Fig. 4). Although oxidation of NADH appears to be surprising, a survey of the literature on SHMT reveals several notable features of the enzyme which support the possibility that NADH can bind to the enzyme. Earlier protocols used for the purification of the enzyme from various sources utilized adsorption and elution of the protein from Blue Sepharose matrix [28,29]. The latter is a matrix of choice for the purification of proteins containing the dinucleotide binding fold. Also, Ramesh and Appaji Rao [30] had shown that Cibacron Blue, a dye that bears structural similarity to NAD, is an inhibitor of SHMT. They also showed that the positive homotropic binding of the substrate, H_4-folate, is altered by interaction with pyridine nucleotides: NAD functions as a negative allosteric effector and NADH as a positive allosteric effector [31]. This is further supported by the presence of alternating α and β strands, a characteristic feature of the dinucleotide binding sites, in the X-ray structure of hSHMT [8], although the classical Rossmann fold observed in the three-dimensional structure of NADH utilizing enzymes such as LDH, etc. is not present in SHMT. In some instances, crystal structures of NADH dependent dehydrogenases exhibit variations of the classical Rossmann fold [32]. However, the observation that NADH oxidation is induced only in H230Y mutant protein and not in any of the other H230 mutant proteins including H230F, lends credence to the hypothesis that the reaction is probably mediated by tyrosyl radicals which have a transient existence. Such a phenomenon, of redox reactions being catalyzed by tyrosyl radicals, has been observed in a number of other enzyme systems and has been extensively investigated in ribonucleotide reductase [33] where a tyrosine (Y122) present in the active center, was shown to participate in the reaction as a free radical. Mutation of this residue to F abolished the activity [34]. Also, in this enzyme, in addition to the tyrosine residue, a thiyl radical was also proposed to be involved in the reaction. An examination of the active site of SHMT does not indicate a residue, which could be functioning between the mutated tyrosine and NADH. A cysteine residue has been implicated in the reaction mechanism of the enzyme but has not been located at the active site. It has been suggested that conformational changes upon substrate binding could position a cysteine residue in the active site [8]. It could be speculated that upon binding of NADH, a similar change could occur and the presence of a cysteine residue and a tyrosyl moiety could facilitate the oxidation at the active site. The alternative hypothesis that NADH oxidation occurs at a different active site into which tyrosine is moved, cannot be ruled out.

The finding that changes in substrate and reaction specificity can be elicited by substitution of single amino-acid residues, are in accord with the recent studies on the evolutionary relationships among pyridoxal*P*-dependent enzymes. Many of these enzymes use homologous protein scaffolds for catalyzing quite diverse reactions [35]. The results presented in this paper clearly point to the induction of an NADH oxidation reaction in SHMT and the role of tyrosine residues in the reaction.

ACKNOWLEDGEMENTS

This work was funded by the Department of Biotechnology, New Delhi, India and the CSIR Emeritus Scientist grant awarded to N. A. R. We thank Prof. T. Ramsarma for critical advise and Ms. Aparna for help with the oxygen consumption studies. Thanks are also due to Mr Jomon Joseph, Mr J. V. Krishna Rao and Ms. Kirthi Narayanswamy for helpful discussions.

REFERENCES

1. Blakely, R.L. (1955) The interconversion of serine and glycine. Participation of pyridoxal-phosphate. *Biochem. J.* **61**, 315–323.
2. Graber, R., Kasper, P., Malashevich, V.N., Sandmeier, E., Berger, P., Gehring, H., Jansonius, J.N. & Christen, P. (1995) Changing the reaction specificity of a pyridoxal-5′-phosphate-dependent enzyme. *Eur. J. Biochem.* **232**, 686–690.
3. Graber, R., Kasper, P., Malashevich, V.N., Strop, P., Gehring, H., Jansonius, J.N. & Christen, P. (1999) Conversion of aspartate aminotransferase into an L-aspartate beta-decarboxylase by a triple active site mutation. *J. Biol. Chem.* **274**, 31203–31208.
4. Vacca, R.A., Christen, P., Malashkevich, V.N., Jansonius, J.N. & Sandmeier, E. (1995) Substitution of apolar residues in the active site of aspartate aminotransferase by histidine- effects on reaction and substrate specificity. *Eur. J. Biochem.* **227**, 481–487.
5. Vacca, R.A., Giannattasio, S., Graber, R., Sandmeier, E., Marra, E. & Christen, P. (1997) Active site Arg→Lys substitutions alter reaction and substrate specificity of aspartate aminotransferase. *J. Biol. Chem.* **272**, 21932–21937.
6. Henderson, G.B., Murgolo, N.J., Kuriyan, J., Osapay, K., Kominos, D., Berry, A., Scrutton, N.S., Hinchliffe, N.W., Perham, R.N. & Cerami, A. (1991) Engineering the substrate specificity of glutathione reductase toward that of trypanothione reduction. *Proc. Natl Acad. Sci. USA* **88**, 8769–8773.
7. Mouratou, B., Kasper, P., Gehring, H. & Christen, P. (1999) Conversion of tyrosine phenol-lyase to dicarboxylic amino acid β-lyase, an enzyme not found in nature. *J. Biol. Chem.* **274**, 1320–1325.
8. Renwick, S.B., Snell, K. & Baumann, U. (1998) The crystal structure of human cytosolic serine hydroxymethyltransferase: a target for cancer chemotherapy. *Structure* **6**, 1105–1116.
9. Hatefi, Y., Talbert, P.T., Osborn, M.J. & Huennekens, F.M. (1959) Tetrahydrofolic acid-5,6,7,8-tetrahydropteroyl-glutamic acid. *Biochem. Prep.* **7**, 89–92.
10. Studier, F.W. & Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113–130.
11. Sambrook, J., Fritisch, E.F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual.* 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
12. Alexander, D.C. (1987). An efficient vector-primer cDNA cloning system. *Methods Enzymol.* **154**, 41–64.
13. Datta, A.K. (1995) Efficient amplification using 'megaprimer' by asymmetric polymerase chain reaction. *Nucleic Acids Res.* **23**, 4530–4531.
14. Jagath-Reddy, J., Appaji Rao, N. & Savithri, H.S. (1996) 3′ non

templated 'A' addition by *Taq* DNA polymerase: an adavntage in the constructon of single and double mutants. *Curr. Sci.* **71**, 710–712.
15. Jagath, J.R., Sharma, B., Bhaskar, B., Datta, A., Appaji Rao, N. & Savithri, H.S. (1997) Importance of the amino terminus in maintenance of oligomeric structure of sheep liver cytosolic serine hydroxymethyltransferase. *Eur. J. Biochem.* **247**, 372–379.
16. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, R.J. (1951) Estimation of proteins by Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
17. Manohar, R., Ramesh, K.S. & Appaji Rao, N. (1982) Purification, physicochemical and regulatory properties of serine hydroxymethyltransferase from sheep liver. *J. Biosci.* **4**, 31–50.
18. Jagath, J.R., Sharma, B., Appaji Rao, N. & Savithri, H.S. (1997) The role of His-134-147, and −150 residues in subunit assembly, cofactor binding, and catalysis of sheep liver cytosolic serine hydroxymethyltransferase. *J. Biol. Chem.* **272**, 24355–24362.
19. Nishikimi, M., Appaji Rao, N. & Yagi, K. (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Comm.* **46**, 849–854.
20. Ravindranath, S.D., Ashok Kumar, A., Prema Kumar, R., Vaidyanathan, C.S. & Appaji Rao, N. (1974) Mechanism of aromatic hydroxylation: properties of a model for pyridine nucleotide dependent flavoprotein hydroxylases. *Arch. Biochem. Biophys.* **165**, 478–484.
21. Holmgren, A. (1989) Thioredoxin and glutaredoxin systems. *J. Biol. Chem.* **264**, 13963–13966.
22. Beinert, H., Holm, R.H. & Munck, E. (1997) Iron-sulphur clusters: nature's modular, multipurpose structures. *Science* **277**, 653–659.
23. Sweaney, V.W. & Rabinowitz, J.C. (1980) Proteins containing 4Fe-4S clusters: an overview. *Ann. Rev. Biochem.* **49**, 139–161.
24. Mishra, P.K. & Drueckhammer, D.G. (1998) Novel cofactor derivatives and cofactor-based models. *Curr. Opin. Chem. Biol.* **6**, 758–765.
25. Chapman, S.K., Welsh, F., Moysey, R., Mowat, C., Doherty, M.K., Turner, K.L., Munro, A.W. & Reid, G.A. (1999) Flavocytochromes: transceivers and relays in biological electron transfer. *Biochem. Soc. Trans.* **27**, 185–189.
26. Perutz, M.F. (1989) Myoglobin and haemoglobin: role of distal residues in reaction with haem ligands. *Trends Biochem. Sci.* **14**, 42–44.
27. Gunsalus, I.C., Lipscomb, J.D., Marshall, V., Frauenfelder, H., Greenbaum, E. & Munck, E. (1972) Structure and reactions of oxygenase active centers: cytochrome P-450 and iron sulfur proteins. *Biochem. Soc. Symposium* **34**, 135–157.
28. Vijaylakshmi, D. (1985) *Purification, regulatory properties, interaction with ligands and active site studies on human liver serine hydroxymethyltransferase*. PhD Thesis. Indian Institute of Science, Bangalore, India.
29. Ramesh, K.S. & Appaji Rao, N. (1978) Regulation of monkey liver serine hydroxymethyltransferase by nicotinamide nucleotide. *Biochem. J.* **174**, 1055–1058.
30. Ramesh, K.S. & Appaji Rao, N. (1980) Inhibition of monkey liver serine hydroxymethyltransferase by Cibacron Blue 3G-A. *Biochem. J.* **187**, 249–252.
31. Ramesh, K.S. & Appaji Rao, N. (1980) Purification and physicochemical, kinetic and immunological properties of allosteric serine hydroxymethyltransferase from monkey liver. *Biochem. J.* **187**, 623–636.
32. Kutzenko, A.S., Lamzin, V.S. & Popov, V.O. (1998) Conserved supersecondary structural motif in NAD-dependent dehydrogenases. *FEBS Lett.* **423**, 105–109.
33. Jordan, A. & Reichard, P. (1998) Ribolnucleotide reductases. *Ann. Rev. Biochem.* **67**, 71–98.
34. Katterle, B., Sahlin, M., Schmidt, P.P., Potsch, S., Logan, D.T., Graslund, A. & Sjoberg, B.M. (1997) Kinetics of transient radicals in *E. coli* ribonucleotide reductase. *J. Biol. Chem.* **272**, 10414–10421.
35. Alexander, F.W., Sandmeier, E., Mehta, P.K. & Christen, P. (1994) Evolutionary relationships among PLP-dependent enzymes Regiospecific α, β and γ families. *Eur. J. Biochem.* **219**, 953–960.