Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 258–261, January 1984
Medical Sciences

Molecular abnormality of an inactive aldehyde dehydrogenase variant commonly found in Orientals

(inactive mutant enzyme/alcohol sensitivity/polymorphism)

AKIRA YOSHIDA, I-YIH HUANG, AND MICHIHARU IKAWA

City of Hope Research Institute, Department of Biochemical Genetics, Duarte, CA 91010

Communicated by Harvey A. Itano, September 19, 1983

ABSTRACT **Usual human livers contain two major aldehyde dehydrogenase [(ALDH) aldehyde:NAD$^+$ oxidoreductase] isozymes—i.e., a cytosolic $ALDH_1$ component and a mitochondrial $ALDH_2$ component—whereas ≈50% of Orientals are "atypical" and have only the $ALDH_1$ isozyme and are missing the $ALDH_2$ isozyme. We previously demonstrated that atypical livers contain an enzymatically inactive but immunologically crossreactive material (CRM) corresponding to the $ALDH_2$ component. The enzymatically active $ALDH_2$ obtained from a usual liver and the CRM obtained from an atypical liver were reduced, *S*-carboxymethylated, and digested by trypsin. Separation of their digests by high-performance reverse-phase chromatography and by two-dimensional paper chromatography and electrophoresis revealed that $ALDH_2$ contained a peptide sequence of -Glu-Leu-Gly-Glu-Ala-Gly-Leu-Gln-Ala-Asn-Val-Gln-Val-Lys- and that the glutamine adjacent to lysine was substituted by lysine in CRM. All other tryptic peptides, including eight peptides containing *S*-carboxymethylcysteine, were common in $ALDH_2$ and CRM. It is concluded that a point mutation in the human $ALDH_2$ locus produced the glutamine → lysine substitution and enzyme inactivation.**

Racial differences in the two major liver enzymes that are involved in alcohol catabolism—i.e., alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) and aldehyde dehydrogenase [(ALDH) aldehyde:NAD$^+$ oxidoreductase, EC 1.2.1.3]—have been found between Caucasians and Orientals. Most Caucasians have the alcohol dehydrogenase isozymes containing the usual β_1 subunit, whereas nearly 90% of Orientals have isozymes with the atypical β_2 subunit (1, 2). The specific activity of the atypical $\beta_2\beta_2$ isozyme is nearly 100 times higher than that of the usual $\beta_1\beta_1$ isozyme (2). It has been shown that the active site cysteine of the usual β_1 subunit is replaced by histidine in the superactive atypical β_2 subunit (2).

Virtually all Caucasians have two major ALDH isozymes—i.e., $ALDH_1$ and $ALDH_2$ in their livers—whereas ≈50% of Orientals are "atypical" in that they have only $ALDH_1$ and are missing $ALDH_2$ (3, 4). A very high incidence (50–80%) of acute alcohol intoxication in Orientals could be related to the absence of active $ALDH_2$ and the presence of superactive $\beta_2\beta_2$ alcohol dehydrogenase isozyme (1, 3). However, the atypical Orientals have an enzymatically inactive but immunologically crossreactive material (CRM) in their livers (5). The amino acid composition, subunit molecular size, and immunological characteristics of CRM were indistinguishable from the corresponding properties of $ALDH_2$ but differed from those of $ALDH_1$ (6). Therefore, CRM should be an abnormal defective protein resulting from a mutation of the $ALDH_2$ locus. Genetic study indicates that two common alleles—i.e., $ALDH^1{}_2$ for the usual $ALDH_2$ isozyme and atypical $ALDH^2{}_2$ for the defective CRM at the same locus—are codominantly expressed in Orientals (7). The present paper reports the molecular difference between the usual $ALDH_2$ and the enzymatically inactive CRM.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

ALDH and CRM. Two ALDH isozymes—i.e., $ALDH_1$ and $ALDH_2$—were purified from autopsy specimens of human liver as described (6). CRM and $ALDH_1$ were purified from atypical Japanese livers, which contained the $ALDH_1$ isozyme but not the active $ALDH_2$ isozyme, as described (6). Purities of the samples were checked by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ as described (6). The proteins were thoroughly dialyzed against water and lyophilized.

***S*-Carboxymethylation and Digestion by Trypsin.** The lyophilized proteins (about 15 mg of each) were denatured, reduced, and *S*-carboxymethylated. The procedure was essentially the same as the one previously described (8), except that guanidine hydrochloride (6 M), instead of urea, was used for denaturation, and [^{3}H]iodoacetate, 0.3 mCi (1 Ci = 3.7×10^{10} Bq), was included in the reagents. The *S*-carboxymethylated protein was digested by trypsin (Sigma, type XI, diphenylcarbamylochloride treated). The protein/trypsin ratio was 30:1 (wt/wt), and the digestion was carried out at 25°C for 8 hr, maintaining the pH at 8.0 with ammonium carbonate. The digests were mixed with acetic acid to adjust the pH to 4.0 and lyophilized.

Peptide Mapping. The tryptic digests, about 4 mg of each, were used for peptide mapping on Whatman 3MM paper. The first dimension was descending chromatography using 1-butanol/acetic acid/H_2O, 4:1:5 (vol/vol) (upper phase), for 18 hr, and the second dimension was electrophoresis using acetic acid/pyridine/H_2O, 1:0.5:100 (vol/vol) (pH 5.1), at 30 V/cm for 90 min. The peptides were stained by 0.03% ninhydrin in acetone. The peptide spots that contained radioactive *S*-carboxymethylcysteine were located in the autoradiogram of the peptide maps.

Separation of Tryptic Peptides by HPLC. The tryptic digests, about 1 mg of each, were dissolved in 100 μl of 0.1% trifluoroacetic acid and separated by reverse-phase HPLC using μBondapak C_{18} (10 μm, 0.39 × 30 cm, Waters Associates). A Spectra Physics (Santa Clara, CA) pump system was used to produce a linear gradient from 0.1% trifluoroacetic acid in H_2O to 0.07% trifluoroacetic acid in 50% H_2O and 50% acetonitrile during a 60-min period at a flow rate of 1 ml/min. Peptides were monitored at 220 nm. Radioactivity of the peptide peaks was measured by a liquid scintillation counter.

Determination of Amino Acid Composition and Amino Acid Sequence. The peptides were eluted from the peptide maps with 6 M HCl or with 2 M acetic acid. The peptides were

Abbreviations: ALDH, aldehyde:NAD$^+$ oxidoreductase; CRM, immunologically crossreactive material.

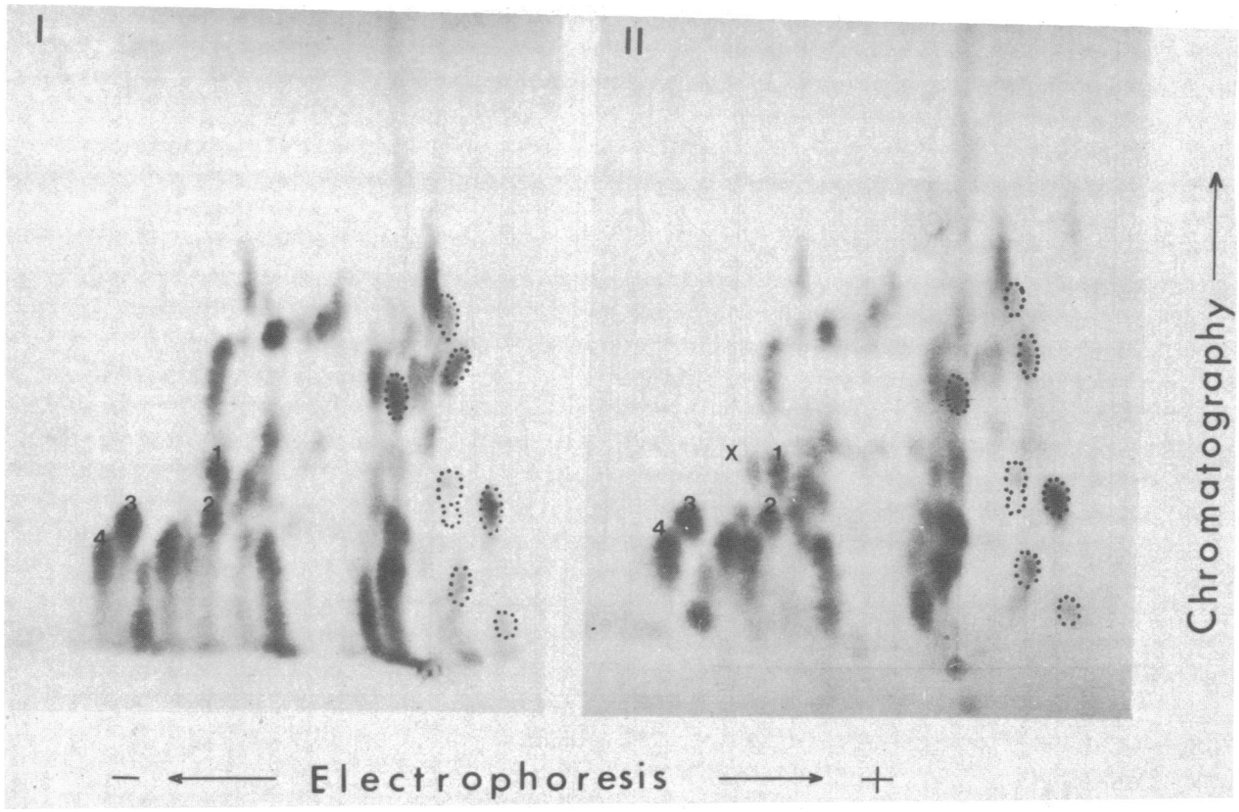

FIG. 1. Peptide maps of tryptic digests of $ALDH_2$ and CRM on Whatman 3MM paper. First dimension: chromatography, 1-butanol/acetic acid/H_2O, 4:1:5 (vol/vol), for 18 hr. Second dimension: electrophoresis, acetic acid/pyridine/H_2O, 1:0.5:100 (vol/vol) (pH 5.1), at 30 V/cm for 90 min. Radioactive spots are encircled. *I*, *S*-carboxymethylated $ALDH_2$; *II*, *S*-carboxymethylated CRM.

hydrolyzed in constant boiling hydrochloric acid for 24 hr at 110°C. Amino acids were determined by a Beckman model 6300 automatic amino acid analyzer. The amino acid sequences of the peptides were determined by manual Edman degradation identifying the products as dansyl amino acids and as phenylthiohydantoin derivatives (9). Dansyl amino acids were identified by thin-layer chromatography, and phenylthiohydantoin derivatives were identified by back hydrolysis with 5.7 M HCl containing 0.1% $SnCl_2$ (10). Phenylthiohydantoin-aspartic acid and phenylthiohydantoin-asparagine were distinguished by thin-layer chromatography (11).

Table 1. Amino acid compositions of tryptic peptides

Peptide	Amino acid, nmol				Presumed sequence
	Alanine	Valine	Lysine	Arginine	
$ALDH_2$					
1	15.5	23.0	26.6	—	Ala-Val-Lys
2	33.6	—	—	19.8	Ala-Ala-Arg
3	—	—	—	22.0	Arg
4	—	—	24.4	—	Lys
CRM					
1	16.8	24.8	29.2	—	Ala-Val-Lys
2	36.1	—	—	21.1	Ala-Ala-Arg
3	—	—	—	28.8	Arg
4	—	—	27.1	—	Lys
X	—	12.2	18.9	—	Val-Lys

Peptide X and several adjacent peptides specified in Fig. 1 were eluted from the peptide maps and analyzed. Amino acids present as <3 nmol are not included. No correction was made for the partial decomposition of NH_2-terminal residues by the ninhydrin reagent used for staining.

RESULTS AND DISCUSSION

Peptide maps of the tryptic digests of *S*-carboxymethylated $ALDH_2$ and CRM were identical except that the spot marked X existing in CRM was absent in $ALDH_2$ (Fig. 1). All eight radioactive peptides—i.e., peptides that contained *S*-carboxymethylcysteine—were common in both proteins. Determination of the amino acid composition and sequence revealed that peptide X was Val-Lys (Table 1).

The elution profiles of the two tryptic digests from the reverse-phase column indicated a clear difference between $ALDH_2$ and CRM. The peak marked A in the $ALDH_2$ profile was absent in the CRM profile, whereas the peak marked C in the CRM profile was absent in the $ALDH_2$ profile (Fig. 2). From the amino acid compositions (Table 2) and the results of Edman degradation of peptide A and peptide C (Fig. 3), the structural difference between $ALDH_2$ and CRM was deduced. Peptide C was two amino acids shorter than peptide A—i.e., missing -Gln-Val- proximal to the COOH-terminal

Table 2. Amino acid compositions of tryptic peptides

Amino acid	Peptide A	Peptide C
Aspartic acid	1.06 (1)	0.95 (1)
Glutamic acid	3.83 (4)	2.64 (3)
Glycine	2.07 (2)	2.02 (2)
Alanine	1.79 (2)	1.85 (2)
Valine	1.94 (2)	1.16 (1)
Leucine	1.83 (2)	2.03 (2)
Lysine	1.00 (1)	1.00 (1)
Total residues	(14)	(12)

The amounts of amino acid residues found in peptide A and peptide C eluted from HPLC are shown, taking that of lysine as 1.00. Amino acids less than 0.3 are not included. The numbers in parentheses are probable molar ratios of amino acids.

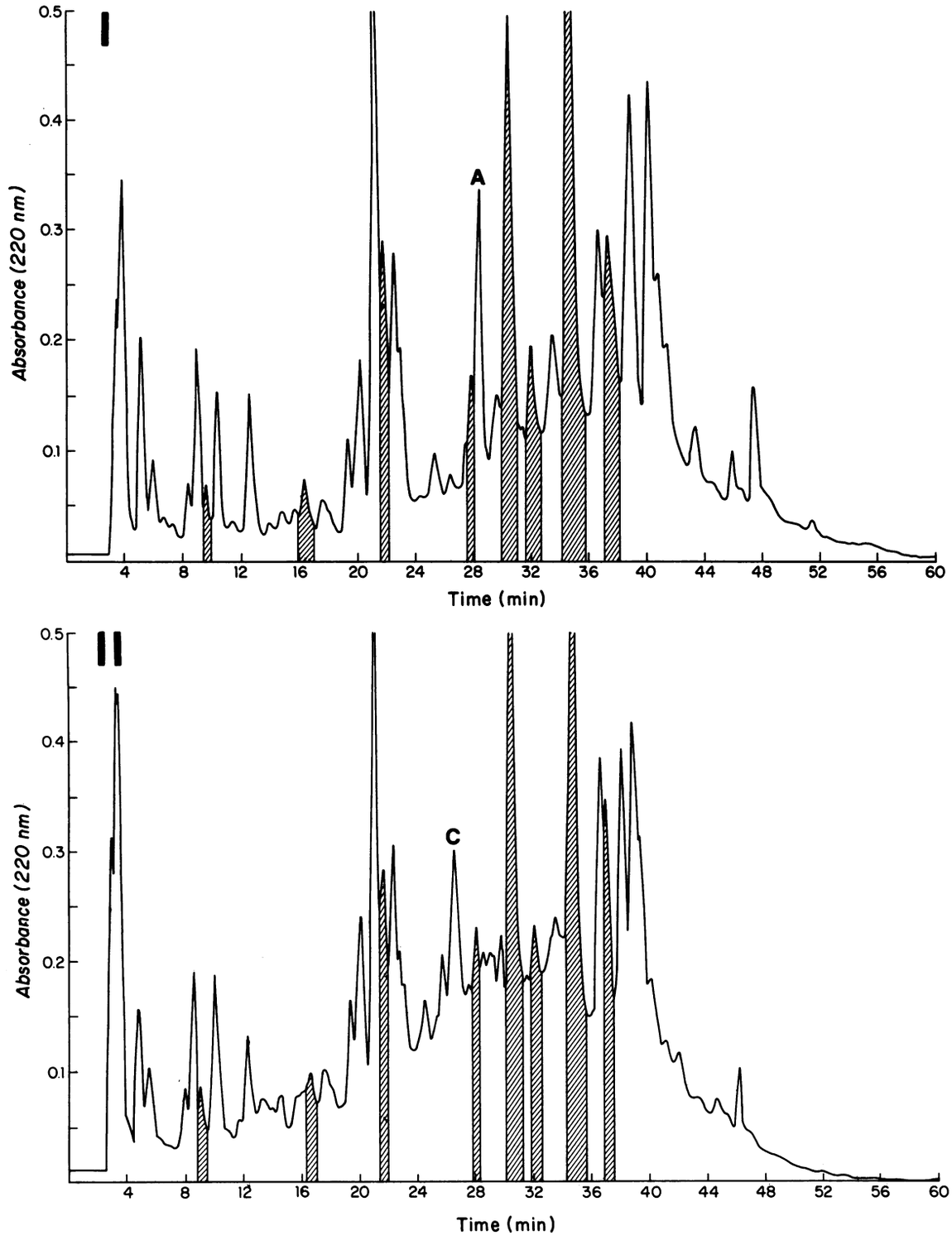

FIG. 2. Elution profiles of tryptic digests of $ALDH_2$ and CRM from a reverse-phase column. The digests (about 1 mg of each) were eluted from a 0.39 × 30 cm μBondapak C_{18} column with a linear gradient from 0.1% trifluoroacetic acid in H_2O to 0.07% trifluoroacetic acid in 50% H_2O and 50% acetonitrile during 60 min at a flow rate of 1 ml/min. Hatched peaks were radioactive. *I*, *S*-carboxymethylated $ALDH_2$; *II*, *S*-carboxymethylated CRM.

lysine. Because the tryptic digests of CRM contained Val-Lys, which was absent in that of $ALDH_2$, one can conclude that the glutamine residue next to valine of $ALDH_2$ was substituted by lysine in CRM. The amino acid substitution found—i.e., glutamine → lysine—is most probably the only abnormality in CRM, although the possibility of the existence of an additional molecular abnormality that could not be detected by the present analysis is not ruled out. The substitution from glutamine to lysine is compatible with the fact that CRM moved slower towards the anode than $ALDH_2$ in electrophoresis (5).

CRM has no ALDH activity and diminished or no affinity for NAD (6). Therefore, the glutamine residue at this specific position must play a crucial role for exhibiting ALDH activi-

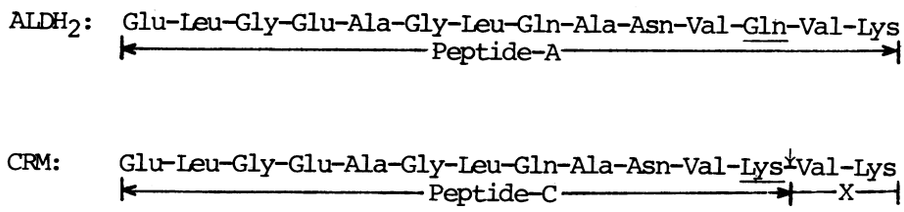

FIG. 3. Structural difference between $ALDH_2$ and CRM. The amino acid sequence of peptide A obtained from $ALDH_2$ and that of peptide C obtained from CRM were determined by manual Edman degradation. The structure of peptide X obtained from CRM was deduced from its amino acid composition. Arrow (↓) shows the trypsin-sensitive bond of the CRM peptide. The positions of the structural difference are underlined.

ty, and the replacement of this glutamine by lysine induces total loss in enzyme activity. It remains unknown whether the glutamine directly participates to form an NAD binding site or whether it plays a role in maintenance of an appropriate three-dimensional structure of the enzyme so that replacement of the glutamine residue by lysine induces conformational changes.

From horse mitochondrial ALDH (corresponding to human $ALDH_2$), Bahr-Lindström *et al.* isolated a tryptic peptide containing cysteine, which was implicated in the coenzyme binding site of the enzyme (12). The present human tryptic peptide with the amino acid substitution has no homology with the horse peptide they obtained, indicating that these peptides are located in different parts of the enzyme molecules. All eight tryptic peptides containing cysteine were common in $ALDH_2$ and CRM (Figs. 1 and 2), indicating that no structural abnormality exists in the vicinity of the active site cysteine of CRM.

Peptide X, Val-Lys, could not be detected by the HPLC analysis (Fig. 2). On the other hand, the peptide substitution—i.e., peptide C in CRM for peptide A in $ALDH_2$—was clearly detected by HPLC analysis but was not observed in the peptide maps. Peptide X was presumably superimposed upon other small peptides in the HPLC profiles, and peptide A and peptide C, which were expected to have very similar charges and hydrophobicities, could not be separated by the two-dimensional paper chromatography and electrophoresis. Two methods—i.e., separation of the peptides by HPLC and peptide mapping on paper—were needed for elucidating the molecular abnormality of the inactive CRM.

All animals thus far reported have two major ALDH isozymes in their livers (13, 14). $ALDH_1$ is cytosolic in origin, has a low K_m for NAD and a high K_m for acetaldehyde, and is inactivated by disulfiram; $ALDH_2$ is mitochondrial in origin, has a high K_m for NAD and a low K_m for acetaldehyde, and is resistant to disulfiram (15, 16). The two isozymes exhibit certain serological homology, indicating that they probably differentiated from a common ancester (6, 17). A point mutation, $\frac{G}{C} \rightarrow \frac{A}{T}$ transition, must have occurred in the human $ALDH_2$ gene, resulting in the glutamine → lysine substitution and the enzyme inactivation. All known mutant enzymes that originated by point mutations retain a certain degree of their original enzyme activities. The mutant $ALDH_2$ with no measurable enzyme activity reported here is unique in this respect. The mutant gene, designated $ALDH^2_2$, is widely spread among Orientals (3, 4). The frequency of the mutant $ALDH^2_2$ gene is estimated to be about 70% and that of the usual $ALDH^1_2$ gene is estimated to be about 30% in Japanese and Chinese. By contrast, the frequency of the mutant gene is found to be very rare in Caucasians and several other ethnic populations (3). The atypical subjects without active $ALDH_2$ enzyme are physiologically normal, except for a possible association with acute alcohol sensitivity (3). Therefore, the mitochondrial $ALDH_2$ appears to be unessential in man, although it might have been essential at certain stages of evolution, and it may still have some physiological role in other animal species. Recently, we found a subject who has $ALDH_2$ isozyme but not $ALDH_1$ isozyme (7). The subject exhibited no physiological abnormality. The cytosolic $ALDH_1$ and the mitochondrial $ALDH_2$ are probably complements to each other, and the presence of either one of these two isozymes may thus fulfill the physiological requirement, at least in man.

This work was supported by Research Grants HL-29515 and AA-05763 from the National Institutes of Health.

1. Stamatoyannopoulos, G., Chen, S.-H. & Fukui, M. (1975) *Am. J. Hum. Genet.* **27**, 780–796.
2. Yoshida, A., Impraim, C. C. & Huang, I.-Y. (1981) *J. Biol. Chem.* **256**, 12430–12436.
3. Goedde, H. W., Harada, S. & Agarwal, D. P. (1979) *Hum. Genet.* **51**, 331–334.
4. Teng, Y.-S. (1981) *Biochem. Genet.* **19**, 107–114.
5. Impraim, C., Wang, G. & Yoshida, A. (1982) *Am. J. Hum. Genet.* **34**, 837–841.
6. Ikawa, M., Impraim, C. C., Wang, G. & Yoshida, A. (1983) *J. Biol. Chem.* **258**, 6282–6287.
7. Yoshida, A., Wang, G. & Davé, V. (1983) *Am. J. Hum. Genet.* **35**, 1117–1125.
8. Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627.
9. Huang, I.-Y., Rubinfien, E. & Yoshida, A. (1980) *J. Biol. Chem.* **255**, 6408–6411.
10. Mendez, E. & Lai, C. Y. (1975) *Anal. Biochem.* **68**, 47–53.
11. Summers, M. R., Smythers, G. W. & Oroszlan, S. I. (1973) *Anal. Biochem.* **53**, 624–628.
12. Bahr-Lindström, H. V., Sohn, S., Woenckhaus, C., Jeck, R. & Jörnvall, H. (1981) *Eur. J. Biochem.* **117**, 521–526.
13. Crow, K. E., Kitson, T. M., MacGibbon, A. K. H. & Batt, R. R. (1974) *Biochim. Biophys. Acta* **350**, 121–128.
14. Eckfeldt, J., Mope, L., Takio, K. & Yonetani, T. (1976) *J. Biol. Chem.* **251**, 236–240.
15. Eckfeldt, J. & Yonetani, T. (1976) *Arch. Biochem. Biophys.* **175**, 717–722.
16. Greenfield, N. J. & Pietruszko, R. (1977) *Biochim. Biophys. Acta* **483**, 35–45.
17. Kitabatake, N., Sasaki, R. & Chiba, H. (1981) *J. Biochem. (Tokyo)* **89**, 1223–1229.